The role of intestinal microbiota in T cell-mediated experimental arthritis

Lessons from the mouse gut

Rebecca L. Rogier

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Colofon

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Chapter 1

General introduction and outline of this thesis

General introduction

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation and progressive destruction of cartilage and bone in multiple joints [1]. This impairs mobility of the joint and causes pain. RA affects 0.5%-1% of the population of the Western world [2]. In joints affected by RA, the synovial membrane is characterized by a hyperplastic lining, pannus formation, and infiltration by innate (macrophages, monocytes, dendritic cells and neutrophils) and adaptive (T cells, B cells and plasma cells) immune cells (Figure 1) [1, 3, 4]. These inflammatory cells produce a variety of cytokines and chemokines which promote cartilage damage, bone erosion and attract more immune cells to the joint [5].

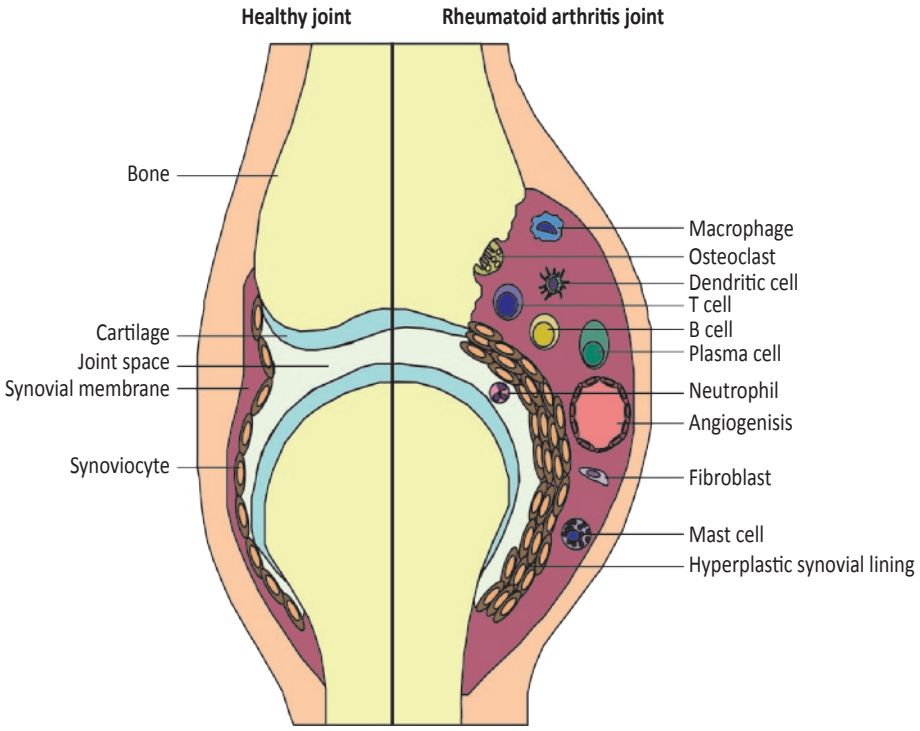


Figure 1. Schematic overview of a healthy synovial joint and an inflamed arthritic joint. A healthy synovial joint (left) and arthritic joint (right). An arthritic joint is characterized by thickening of the synovial lining and influx of inflammatory cells. The invading cells promote cartilage degradation and bone erosion through the production of inflammatory and destructive mediators (adapted from [6]).

Although the exact etiology is unknown, RA is considered to be a multifactorial disease driven by a combination of genetic and environmental factors[7]. Recent studies have revealed multiple genetic susceptibility loci involved in RA (e.g., HLA-DRB1, PTPN22, STAT4, CTLA-4) [8, 9]. Several of these susceptibility alleles are known to affect activation and regulation of the immune system [10]. However, the presence of genetic susceptibility factors is neither necessary nor sufficient for RA development, underlining a critical role for environmental factors.
The development of autoimmunity in RA starts several years before clinical symptoms of the disease are present [11-13]. Absence of synovitis in this pre-clinical phase suggests that the disease is initiated at extra-articular sites. Mucosal surfaces have recently been proposed as a site of initiation of autoimmunity and the induction of autoantibodies in the preclinical phase of RA [14-18]. For instance, smoking and the subsequent citrullination of peptides have been suggested to give rise to the development of anti-citrullinated protein antibodies (ACPAs) thereby contributing to RA development [19-21]. In addition, microorganisms residing at mucosal surfaces such as the lung [14-16], periodontal [22-26] and intestinal mucosal tissues [26-29] have been implicated in the development of RA. The focus of this thesis was to dissect the relevance of intestinal microbiota and intestinal mucosal immunity in the development of arthritis.

Intestinal microbiota and RA

The gastrointestinal tract, skin, oral cavity, respiratory tract and female genital tract are all colonized by microorganism; all these microorganisms (and their genomes) together are known as the microbiome [30]. The intestinal tract is the most densely populated mucosal site of the body, especially containing large amounts of bacteria [31]. Several studies reported that the diversity and composition of intestinal microbiota are disturbed in patients with new-onset as well as chronic RA [26, 27, 29, 32]. Two studies reported significant overrepresentation of *Prevotella copri* in patients with new-onset RA prior to immunosuppressive treatment [27, 32]. In addition, increased *Prevotella* abundance in RA patients was accompanied by a reducted abundance of *Bacteroides* [27]. Another study found *Lactobacillus salivarius* to be significantly expanded in RA fecal microbiota, especially in patients with very active disease [26]. A fourth study in patients with longstanding, treated RA demonstrated increased abundance of *Collinsella*, *Eggerthella*, and *Faecalibacterium* [29]. Furthermore, fecal microbiota of RA patients was shown to contain significantly more *Lactobacillus* compared to microbiota of healthy controls [33]. These studies clearly show that the composition of the intestinal microbiome is perturbed in patients with RA. However, the functional relevance and the mechanistic links between these perturbations and the immune pathways driving RA pathogenesis are not well understood.

Intestinal microbiota and the immune system

The intestinal immune system must establish an appropriate balance between tolerance to the gut commensal microbiota and defense against pathogens [34, 35]. Intestinal epithelial cells (IEC), connected to each other by tight junctions, provide a physical barrier between the lumen and the underlying lamina propria (Figure 2) [36]. Specialized epithelial cells, called goblet cells, produce a thick mucus layer which limits direct contact between bacteria and IEC (Figure 2) [37]. This mucus layer contains antimicrobial peptides (AMPs), produced by goblet cells, enterocytes and Paneth cells, and large amounts of secretory immunoglobulin A (IgA) produced by plasma cells [38-40]. Most AMPs kill bacteria directly through enzymatic attack of the bacterial cell wall or by disrupting the inner membrane [35]. Secreted IgA binds to bacteria which promotes clearance and limits their association with the epithelium, entrapping them in mucus [41]. Underneath the epithelium lies the lamina propria, a connective tissue that contains various immune cells including macrophages, dendritic cells (DCs), T cells and plasma cells (Figure 2). Bacteria that breach the IEC barrier are normally phagocytosed and eliminated by lamina propria macrophages [42]. Specialized epithelial cells in Peyer's patches, called microfold cells (M cells), sample the lumen and present antigens to underlying mucosal immune cells (Figure 2) [43]. In addition, DCs in Peyer's patches and lamina propria can sense and actively sample the intestinal content and present this to nearby T cells (Figure 2)[44, 45]. Moreover, Toll-like receptors (TLRs) on macrophages and DCs recognize microbe-associated molecular patterns (MAMPs), which are shared by many microorganisms [46]. Activation of TLRs on antigen presenting cells (APCs) induces the production of cytokines and expression of co-stimulatory signals which will activate naive T cells [47]. After the activation of naive T cells by APCs, the cytokine environment determines the direction of the T helper (Th) cell differentiation (Figure 3). Th1 cells are induced by interleukin (IL)-12 and produce high levels of interferon γ (IFNγ), thereby activating macrophages against intracellular pathogens (Figure 3) [49, 50]. Th2 cells are induced by IL-4 and produce IL-4, IL-5 and IL-13 to activate eosinophils which offer protection against parasitic infections (Figure 3) [49, 51]. The differentiation of Th17 cells and regulatory T (Treg) cells is closely linked as both require transforming growth factor β (TGFβ) [52]. When TGFβ is present in combination with IL-6 or IL-21, naive T cells will differentiate into Th17 cells (Figure 3) [52, 53]. However, in the absence of IL-6 and IL-21, TGFβ drives differentiation of naive T cells into immune-regulating Treg cells (Figure 3) [52]. In addition, IL-23 is known to be important for the expansion and survival of Th17 cells [53]. Th17 cells produce cytokines such as IL-17A, IL-17F, IL-21, IL-22 and GM-CSF and are involved in clearance of extracellular bacteria and fungi (Figure 3) [54]. Treg cells produce IL-10 and TGFβ thereby dampening inflammation after microbial infection (Figure 3).

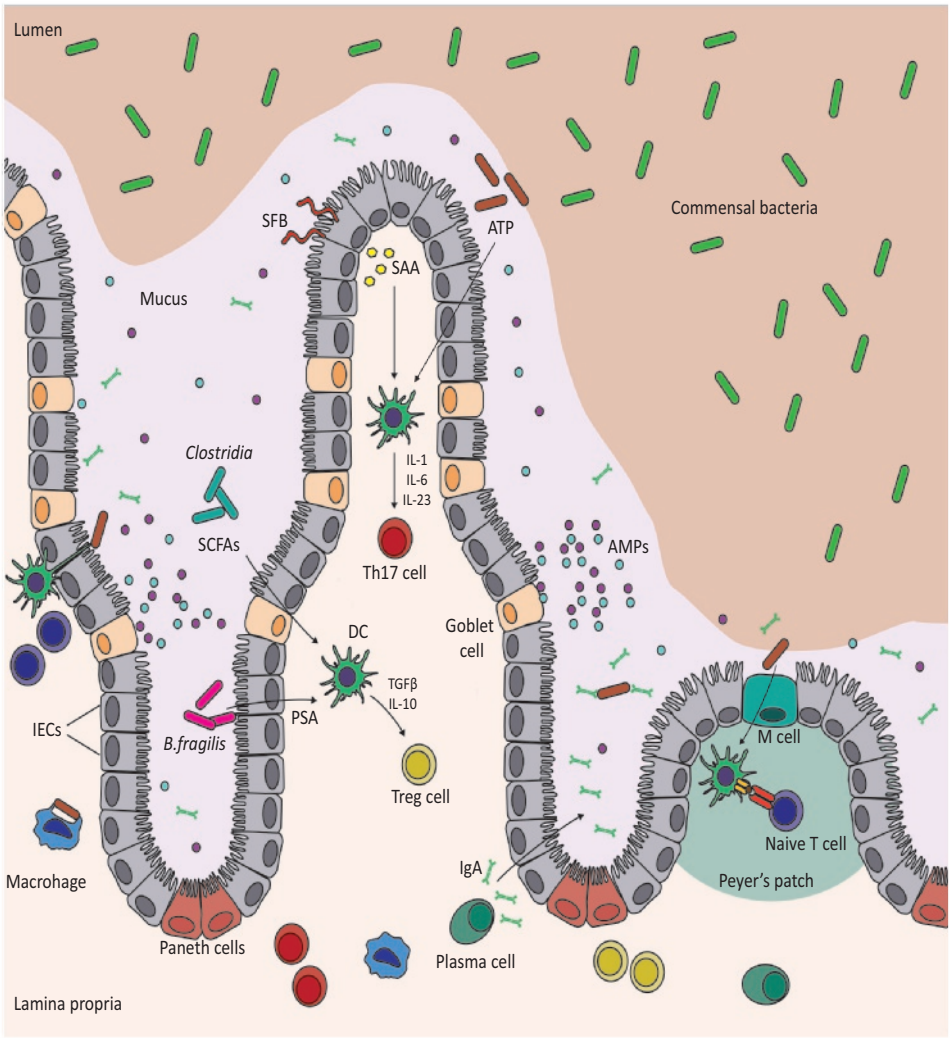


Figure 2. The structure of the intestinal mucosa. The epithelium consists of a single layer of intestinal epithelial cells (IECs) and provides a physical barrier between lumen and lamina propria. The epithelium is covered by a mucus layer (produced by goblet cells) containing antimicrobial peptides (AMPs) and secretory immunoglobulin A (IgA). Underneath the epithelium lies the lamina propria that contains various immune cells including macrophages, dendritic cells (DCs), T cells and IgA secreting plasma cells. Bacteria that breach the epithelial barrier are phagocytosed and eliminated by macrophages. Bacteria-derived products such as polysaccharide A (PSA) from Bacteroides fragilis and short chain fatty acids (SCFAs) produced by Clostridia bacteria can promote the induction of colonic Treg cells. In contrast, segmented filamentous bacteria (SFB) drive the differentiation of intestinal Th17 cells through induction of serum amyloid A (SAA) production by IECs. Bacteria-derived ATP promotes intestinal Th17 differentiation as well. Microfold cells (M cells) in Peyer's patches transfer bacterial antigens across the epithelium to antigen presenting cells such as DCs, which will present the antigen to T cells. In addition, DCs in the lamina propria can directly sense and take-up intestinal content and present this to nearby T cells (Adapted from [48]).

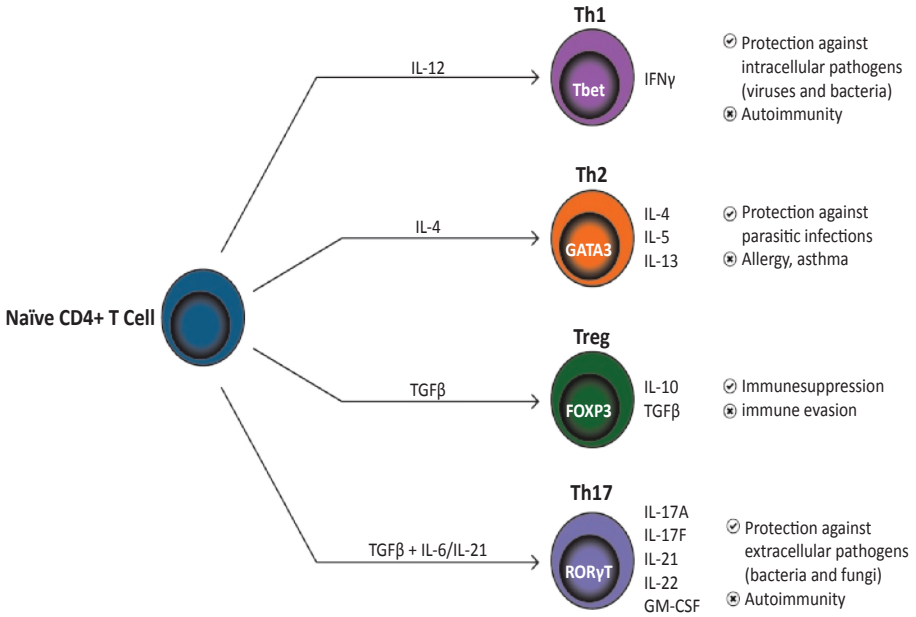


Figure 3. Differentiation of T helper cell subsets. Following activation by antigen-presenting cells, naive CD4+ T cells can differentiate into different effector Th cell subsets: T helper 1 (Th1), Th2, Th17 and regulatory T (Treg) cells. This depends on the local cytokine environment that activates the lineage determining transcription factors, i.e., T-bet for Th1 cells, GATA3 for Th2 cells, forkhead box P3 (FOXP3) for Treg cells and Retinoic acid receptor-related orphan receptor γt (RORγt) for Th17 cells.

Some commensal bacteria can actively induce Treg proliferation thereby inducing immune tolerance [55, 56]. For instance, intestinal colonization with *Bacteroides fragilis* or with a mixture of *Clostridia* strains was shown to promote intestinal Treg cell differentiation in mice [57, 58] (Figure 2). The effect of *B. fragilis* on Treg cell development is mediated by capsular polysaccharide A (PSA), while *Clostridia* induce differentiation of colonic T cells through production of high levels of short chain fatty acids (SCFAs) [59-62]. In contrast, segmented filamentous bacteria (SFB) potently induce Th17 differentiation in intestinal lamina propria of mice [63-65] (Figure 2). In response to SFB, IECs produce serum amyloid A (SAA) 1 and SAA2 which promotes Th17 differentiation and local IL-17 production [63, 66, 67]. SFB induced epithelial SAA production was shown to require both IL-23 and IL-22 [66]. In addition, bacteria-derived ATP is known to promote intestinal Th17 differentiation as well [68] (Figure 2). This shows that intestinal microbiota play an important role in defining the balance between pro- and anti-inflammatory Th cells, thereby preserving intestinal homeostasis. Dysbiosis of the intestinal microbiota is thought to contribute to the disturbed immune response and intestinal inflammation observed in inflammatory bowel disease [69, 70].

Interestingly, several studies in mice suggest that the intestinal microbiota not only affects intestinal Th cell balances, but also affects systemic Th cell activation and the development of T cell-driven autoimmune processes at sites distal from the gut [28, 65, 71-74].

Intestinal microbiota and systemic autoimmunity

Studies in germ-free (GF) mice have shown that the development of experimental autoimmune encephalomyelitis (EAE, a model for multiple sclerosis) and autoimmune arthritis depends on the presence of microbiota, as these diseases are strongly attenuated in GF mice [28, 65, 74, 75]. Intestinal as well as systemic Th17 differentiation was strongly reduced in these GF mice [65, 74]. Interestingly, mono-colonization of GF mice with SFB induced the differentiation of intestinal Th17 cells and rapid onset of disease [65, 74]. In addition, treating mice with broad-spectrum antibiotics impaired the development of EAE [73]. Furthermore, colonization of mice with *B. fragilis* protected mice against EAE, a process that was shown to depend on the presence of PSA in the bacterial capsule which increased numbers of Treg cells in cervical lymph nodes via TLR2 [76, 77]. In addition, it was shown that inoculating GF arthritis-prone SKG mice with *Prevotella*-dominated microbiota of RA patients resulted in increased intestinal and systemic Th17 cell responses and enhanced the development of arthritis compared with mice receiving fecal microbiota from healthy controls [28]. Another study showed that colonizing mice with the human gut commensal *Prevotella histicola* suppressed Th17 responses and the development of collagen-induced arthritis (CIA) [78]. In contrast, treating mice with *Collinsella aerofaciens* after induction of CIA increased disease incidence and severity [29]. It has been shown that interleukin-1 receptor antagonist (IL-1Ra) deficient mice that spontaneously develop severe arthritis are protected under GF condition and by additional TLR4 deficiency associated with reduced systemic Th17 response [75]. These observations suggest that microbiota-induced modulation of intestinal Th cell differentiation may modulate systemic Th cells and the development of autoimmune diseases such as arthritis. However, the role of these microbiota-induced T cells in the pathogenesis of arthritis is unknown.

T helper cells in rheumatoid arthritis

As mentioned before, the microbiota has a profound effect on the balance between the pro-inflammatory Th1 and Th17 cells and the protective Treg cells, both at mucosal surfaces and systemically [31, 72]. Considerable evidence supports an important role for Th cells in RA pathology [79, 80]. First of all, T cells are one of the most abundant cell types in RA synovium, with the majority being $CD4^+$ [81]. Secondly, several genetic polymorphisms known to predispose to RA (*e.g.*, HLA-DRB1, CTLA-4, CD40, CD28 and PTPN22) are known to affect the activation of T cells [7]. The therapeutic efficacy of Abatacept, which targets the

co-stimulatory signalling between APCs and T cells, further supports the role of Th cells in RA pathogenesis [82]. Furthermore, studies in animal models showed that experimental arthritis can be induced by transferring Th cells from an arthritic animal to a naive mouse [83, 84].
In recent years, many animal and clinical studies have focussed on the role of IL-17 in RA. In mice, blocking of IL-17A and the IL-17 receptor resulted in significant reduction of experimental arthritis [85, 86]. Despite promising results in mice, targeting IL-17 in RA patients was somewhat disappointing; however, it did result in a significant improvement in a subset of patients [87-89]. Furthermore, in the humanized RA synovium-SCID model, it was demonstrated that anti-IL-17 treatment was only effective when the synovial tissue contained high numbers of T cells [90]. This suggests that IL-17 blocking could be effective in a subset of patients. Moreover, the role of Th17 cells in RA is likely to extend beyond that of IL-17 alone, as Th17 cells produce several other proinflammatory cytokines such as IL-22, tumor-necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which are known to contribute to inflammation, cartilage destruction and osteoclastogenesis [91-95]. However, previous studies in animal models and clinical trials only focused on IL-17 and therefore further studies to unravel the role of Th17 cells in the pathogenesis of arthritis are required.

Aim and outline of this thesis

Several studies have shown that the intestinal microbiome is disturbed in patients with new-onset and chronic autoimmune inflammatory arthritis. Recent studies in mouse models suggest that development and progression of arthritis is highly affected by the intestinal microbiome. However, the mechanistic link between the microbiota and the immune pathways driving arthritis pathology is not well understood. The general aim of this thesis was to advance our understanding of the involvement of commensal intestinal microbiota in experimental arthritis and to identify the microbiome-induced innate and adaptive immune pathways that affect arthritis development and progression.

In Chapter 2, current evidence supporting the involvement of commensal intestinal microbiota in RA, along with a potential role of TLRs in modulating the relevant Th cell responses, is reviewed.

In Chapter 3, we investigated the role of a physiologic cytokine inhibitor, IL-1Ra, in regulation of commensal intestinal microbiota. In addition, we assessed the involvement of microbiota subsets and innate and adaptive mucosal immune responses that underlie the development of spontaneous arthritis in IL-1Ra deficient mice. We show that IL-1Ra plays a critical role in maintaining the natural

diversity and composition of intestinal microbiota. In addition, our research results suggest a role for tobramycin-sensitive intestinal microbiota and TLR4 activation in mucosal Th17 cell induction associated with the development of autoimmune arthritis in mice.

In Chapter 4, we investigated whether alterations of the intestinal microbiota precede clinical disease. We show that the intestinal microbial community undergoes marked changes in the preclinical phase of CIA. In addition, we show that the abundance of intestinal Th17 cells correlates with arthritis severity and that elimination of the intestinal microbiota during established arthritis specifically reduces intestinal Th17 cells and attenuates arthritis.

In Chapter 5, we investigated whether mucosal immune activation, specifically intestinal Th17 cell differentiation, occurs before the clinical onset of arthritis, and we examined the requirement of these Th17 cells for the development of arthritis. We show that immunization with collagen type II induces a robust T cell response that precedes the onset of collagen-induced arthritis. We further examined the involvement of Th17 cells in arthritis pathogenesis using conditional Th17-deficient (CD4-Cre^{+} Rorc$^{flox/flox}$) mice. We show that Th17 cells only mediate the pathogenesis of arthritis in the context of specific Th17-inducing microbiota.

In Chapter 6, we assessed the therapeutic potential of dietary supplementation with a prebiotic mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) in experimental arthritis in mice. We show that this prebiotic diet has a pronounced effect on the composition of the fecal microbiota. Furthermore, we observed a significant increase in the bone mineral density in mice upon dietary supplementation with scGOS/lcFOS. However, the scGOS/lcFOS-induced alterations of the intestinal microbiota did not significantly affect the intestinal and systemic T helper cell subsets and were not sufficient to reproducibly suppress arthritis in mice.

Finally, in Chapter 7 the results of this thesis are summarized and the implications of the results are discussed.

References

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003;423(6937):356-61.
2. Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. Arthritis research. 2002;4 Suppl 3:S265-72.
3. Henderson B, Revell PA, Edwards JC. Synovial lining cell hyperplasia in rheumatoid arthritis: dogma and fact. Annals of the rheumatic diseases. 1988;47(4):348-9.
4. Yanni G, Whelan A, Feighery C, Bresnihan B. Analysis of cell populations in rheumatoid arthritis synovial tissues. Seminars in arthritis and rheumatism. 1992;21(6):393-9.
5. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet (London, England). 2016;388(10055):2023-38.
6. Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. Nature reviews Drug discovery. 2003;2(6):473-88.
7. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19.
8. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. Nature. 2014;506(7488):376-81.
9. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nature genetics. 2010;42(6):508-14.
10. McInnes IB, Schett G. Pathogenetic insights from the treatment of rheumatoid arthritis. Lancet (London, England). 2017;389(10086):2328-37.
11. van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, et al. Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis and rheumatism. 2011;63(11):3226-33.
12. Deane KD, O'Donnell CI, Hueber W, Majka DS, Lazar AA, Derber LA, et al. The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner. Arthritis and rheumatism. 2010;62(11):3161-72.
13. van der Woude D, Rantapaa-Dahlqvist S, Ioan-Facsinay A, Onnekink C, Schwarte CM, Verpoort KN, et al. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. Annals of the rheumatic diseases. 2010;69(8):1554-61.
14. Scher JU, Joshua V, Artacho A, Abdollahi-Roodsaz S, Ockinger J, Kullberg S, et al. The lung microbiota in early rheumatoid arthritis and autoimmunity. Microbiome. 2016;4(1):60.
15. Catrina AI, Ytterberg AJ, Reynisdottir G, Malmstrom V, Klareskog L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. Nature reviews Rheumatology. 2014;10(11):645-53.
16. Chatzidionisyou A, Catrina AI. The lung in rheumatoid arthritis, cause or consequence? Current opinion in rheumatology. 2016;28(1):76-82.
17. Rosenbaum JT, Asquith MJ. The Microbiome: a Revolution in Treatment for Rheumatic Diseases? Current rheumatology reports. 2016;18(10):62.
18. Scher JU, Abramson SB. The microbiome and rheumatoid arthritis. Nature reviews Rheumatology. 2011;7(10):569-78.
19. Klareskog L, Padyukov L, Alfredsson L. Smoking as a trigger for inflammatory rheumatic diseases. Current opinion in rheumatology. 2007;19(1):49-54.
20. Klareskog L, Stolt P, Lundberg K, Kallberg H, Bengtsson C, Grunewald J, et al. A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. Arthritis and rheumatism. 2006;54(1):38-46.

20. Makrygiannakis D, Hermansson M, Ulfgren AK, Nicholas AP, Zendman AJ, Eklund A, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. Annals of the rheumatic diseases. 2008;67(10):1488-92.
21. Scher JU, Ubeda C, Equinda M, Khanin R, Buischi Y, Viale A, et al. Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. Arthritis and rheumatism. 2012;64(10):3083-94.
22. Mikuls TR, Payne JB, Yu F, Thiele GM, Reynolds RJ, Cannon GW, et al. Periodontitis and Porphyromonas gingivalis in patients with rheumatoid arthritis. Arthritis & rheumatology (Hoboken, NJ). 2014;66(5):1090-100.
23. Payne JB, Golub LM, Thiele GM, Mikuls TR. The Link Between Periodontitis and Rheumatoid Arthritis: A Periodontist's Perspective. Current oral health reports. 2015;2:20-9.
24. Quirke AM, Lugli EB, Wegner N, Hamilton BC, Charles P, Chowdhury M, et al. Heightened immune response to autocitrullinated Porphyromonas gingivalis peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis. Annals of the rheumatic diseases. 2014;73(1):263-9.
25. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med. 2015;21(8):895-905.
26. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife. 2013;2:e01202.
27. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine. Arthritis & rheumatology (Hoboken, NJ). 2016;68(11):2646-61.
28. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome medicine. 2016;8(1):43.
29. Lederberg J. Infectious history. Science (New York, NY). 2000;288(5464):287-93.
30. Kamada N, Nunez G. Regulation of the immune system by the resident intestinal bacteria. Gastroenterology. 2014;146(6):1477-88.
31. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T cells in the intestine. Arthritis & rheumatology (Hoboken, NJ). 2016.
32. Liu X, Zou Q, Zeng B, Fang Y, Wei H. Analysis of fecal Lactobacillus community structure in patients with early rheumatoid arthritis. Current microbiology. 2013;67(2):170-6.
33. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature reviews Immunology. 2004;4(6):478-85.
34. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nature reviews Immunology. 2010;10(3):159-69.
35. Shen L, Turner JR. Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed. American journal of physiology Gastrointestinal and liver physiology. 2006;290(4):G577-82.
36. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(39):15064-9.
37. Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson LG, Midtvedt T, Putsep K, et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. Gut. 2008;57(6):764-71.
38. Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, et al. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(7):1981-6.
39. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science (New York, NY). 2004;303(5664):1662-5.

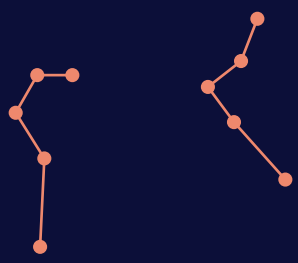
41. Mantis NJ, Rol N, Corthesy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal immunology. 2011;4(6):603-11.
42. Kelsall B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. Mucosal immunology. 2008;1(6):460-9.
43. Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunol. 2013;6(4):666-77.
44. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science. 2005;307(5707):254-8.
45. Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, et al. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 2013;38(3):581-95.
46. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. International reviews of immunology. 2011;30(1):16-34.
47. Pasare C, Medzhitov R. Toll-like receptors and acquired immunity. Seminars in immunology. 2004;16(1):23-6.
48. Muniz LR, Knosp C, Yeretssian G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. Frontiers in immunology. 2012;3:310.
49. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med. 2009;361(9):888-98.
50. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science (New York, NY). 2001;292(5523):1907-10.
51. Charles N, Watford WT, Ramos HL, Hellman L, Oettgen HC, Gomez G, et al. Lyn kinase controls basophil GATA-3 transcription factor expression and induction of Th2 cell differentiation. Immunity. 2009;30(4):533-43.
52. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.
53. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24(2):179-89.
54. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity. 2008;28(4):454-67.
55. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. The Journal of experimental medicine. 2007;204(8):1775-85.
56. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. The Journal of experimental medicine. 2007;204(8):1757-64.
57. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science (New York, NY). 2011;331(6015):337-41.
58. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A. 2010;107(27):12204-9.
59. Narushima S, Sugiura Y, Oshima K, Atarashi K, Hattori M, Suematsu M, et al. Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia. Gut microbes. 2014;5(3):333-9.
60. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell host & microbe. 2012;12(4):509-20.

61. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science (New York, NY). 2013;341(6145):569-73.
62. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50.
63. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485-98.
64. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009;31(4):677-89.
65. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
66. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. Cell. 2015;163(2):381-93.
67. Ivanov, II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell host & microbe. 2008;4(4):337-49.
68. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, et al. ATP drives lamina propria T(H)17 cell differentiation. Nature. 2008;455(7214):808-12.
69. Becker C, Neurath MF, Wirtz S. The Intestinal Microbiota in Inflammatory Bowel Disease. ILAR journal. 2015;56(2):192-204.
70. Sartor RB, Wu GD. Roles for Intestinal Bacteria, Viruses, and Fungi in Pathogenesis of Inflammatory Bowel Diseases and Therapeutic Approaches. Gastroenterology. 2017;152(2):327-39. e4.
71. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science (New York, NY). 2012;336(6086):1268-73.
72. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75-84.
73. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. Journal of immunology (Baltimore, Md : 1950). 2009;183(10):6041-50.
74. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4615-22.
75. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. The Journal of clinical investigation. 2008;118(1):205-16.
76. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, et al. Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. Journal of immunology (Baltimore, Md : 1950). 2010;185(7):4101-8.
77. Ochoa-Reparaz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. Mucosal immunology. 2010;3(5):487-95.
78. Marietta EV, Murray JA, Luckey DH, Jeraldo PR, Lamba A, Patel R, et al. Suppression of Inflammatory Arthritis by Human Gut-Derived Prevotella histicola in Humanized Mice. Arthritis & rheumatology (Hoboken, NJ). 2016;68(12):2878-88.

79. Isaacs JD. Therapeutic T-cell manipulation in rheumatoid arthritis: past, present and future. Rheumatology (Oxford, England). 2008;47(10):1461-8.
80. Goronzy JJ, Weyand CM. T-cell regulation in rheumatoid arthritis. Current opinion in rheumatology. 2004;16(3):212-7.
81. Zvaifler NJ, Boyle D, Firestein GS. Early synovitis--synoviocytes and mononuclear cells. Seminars in arthritis and rheumatism. 1994;23(6 Suppl 2):11-6.
82. Kremer JM, Russell AS, Emery P, Abud-Mendoza C, Szechinski J, Westhovens R, et al. Long-term safety, efficacy and inhibition of radiographic progression with abatacept treatment in patients with rheumatoid arthritis and an inadequate response to methotrexate: 3-year results from the AIM trial. Annals of the rheumatic diseases. 2011;70(10):1826-30.
83. Horai R, Nakajima A, Habiro K, Kotani M, Nakae S, Matsuki T, et al. TNF-alpha is crucial for the development of autoimmune arthritis in IL-1 receptor antagonist-deficient mice. The Journal of clinical investigation. 2004;114(11):1603-11.
84. Kadowaki KM, Matsuno H, Tsuji H, Tunru I. CD4+ T cells from collagen-induced arthritic mice are essential to transfer arthritis into severe combined immunodeficient mice. Clinical and experimental immunology. 1994;97(2):212-8.
85. Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova FE, et al. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. Am J Pathol. 2005;167(1):141-9.
86. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis and rheumatism. 2004;50(2):650-9.
87. Genovese MC, Greenwald M, Cho CS, Berman A, Jin L, Cameron GS, et al. A phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. Arthritis & rheumatology (Hoboken, NJ). 2014;66(7):1693
88. Burmester GR, Durez P, Shestakova G, Genovese MC, Schulze-Koops H, Li Y, et al. Association of HLA-DRB1 alleles with clinical responses to the anti-interleukin-17A monoclonal antibody secukinumab in active rheumatoid arthritis. Rheumatology (Oxford, England). 2016;55(1):49-
89. Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Aelion JA, et al. One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study. The Journal of rheumatology. 2014;41(3):414-21.
90. Koenders MI, Marijnissen RJ, Joosten LA, Abdollahi-Roodsaz S, Di Padova FE, van de Loo FA, et al. T cell lessons from the rheumatoid arthritis synovium SCID mouse model: CD3-rich synovium lacks response to CTLA-4Ig but is successfully treated by interleukin-17 neutralization. Arthritis and rheumatism. 2012;64(6):1762-70.
91. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. Nature reviews Rheumatology. 2015;11(7):415-29.
92. Kim KW, Kim HR, Park JY, Park JS, Oh HJ, Woo YJ, et al. Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts. Arthritis and rheumatism. 2012;64(4):1015-23.
93. Avci AB, Feist E, Burmester GR. Targeting GM-CSF in rheumatoid arthritis. Clinical and experimental rheumatology. 2016;34(4 Suppl 98):39-44.
94. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. Nature reviews Rheumatology. 2016;12(1):37-48.
95. Leipe J, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, et al. Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis. Annals of the rheumatic diseases. 2011;70(8):1453-7.

Chapter 2

Toll-like receptor mediated modulation of T cell response by commensal intestinal microbiota as a trigger for autoimmune arthritis



Chapter 2

Toll-like receptor mediated modulation of T cell response by commensal intestinal microbiota as a trigger for autoimmune arthritis

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Abstract

In autoimmune diseases, a disturbance of the balance between T helper 17 (Th17) and regulatory T cells (Tregs) is often observed. This disturbed balance is also the case in rheumatoid arthritis (RA). Genetic predisposition to RA confers the presence of several polymorphisms mainly regulating activation of T lymphocytes. However, the presence of susceptibility factors is neither necessary nor sufficient to explain the disease development, emphasizing the importance of environmental factors. Multiple studies have shown that commensal gut microbiota is of great influence on immune homeostasis and can trigger the development of autoimmune diseases by favoring induction of Th17 cells over Tregs. However the mechanism by which intestinal microbiota influences the Th cell balance is not completely understood. Here we review the current evidence supporting the involvement of commensal intestinal microbiota in rheumatoid arthritis, along with a potential role of Toll-like receptors (TLRs) in modulating the relevant Th cell responses to trigger autoimmunity. A better understanding of TLR triggering by intestinal microbiota and subsequent T cell activation might offer new perspectives for manipulating the T cell response in RA patients and may lead to the discovery of new therapeutic targets or even preventive measures.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, which is characterized by chronic inflammation and progressive cartilage and bone destruction in multiple joints. A world-wide prevalence of about 1% ranks RA among the most-common autoimmune disorders [1]. Current therapy of RA is based on a choice, or often a combination, of non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids and recently the so-called Biologicals targeting specific cytokines or certain immune cells.

The etiopathology of RA is complex, because cells of the innate and adaptive immune system as well as joint resident cells such as fibroblasts and chondrocytes contribute to the development and progression of RA [2]. The production of proinflammatory cytokines such as tumor necrosis factor (TNF) α and interleukin (IL)-1 and activation of lymphocytes are considered to play important roles in RA pathogenesis [3, 4]. A specific subset of T cells, known as T helper 17 (Th17) cells, is considered to be a major pathogenic mediator in RA [3, 5, 6].

Although the exact etiology remains unclear to date, RA is generally considered a multifactorial disease in which both genetic and environmental factors play a role [7]. Epidemiological studies have revealed that the largest genetic risk factors for RA are certain alleles of the HLA-DR gene [8]. In addition, polymorphisms in protein tyrosine phosphatase N22 (PTPN22), peptidyl arginine deiminase type IV (PADI4), signal transducer and activator of transcription 4 (STAT4) and TNF receptor-associated factor 1/complement C5 (TRAF1/C5) were found associated with RA [8]. However, the presence of susceptibility factors is neither necessary nor sufficient to explain the disease development, underlining a critical role for environmental factors.

Meta-analysis has shown that smoking is one of the environmental factors associated with RA pathogenesis [9]. In addition to smoking, periodontal pathogens such as *Porphyromonas gingivalis* and the induced periodontal disease have been implicated in the etiology of RA [10, 11]. Besides infectious bacteria, commensal bacteria have been implicated in the pathogenesis of RA [12]. In addition, there is strong evidence that Toll-like receptors (TLRs), which recognize microbial products, contribute to RA progression [13-15].

Most of the polymorphisms associated with RA are involved in regulating T cell activation [16]. The genetically altered T cells are potentially auto-reactive, i.e. they may react to self-antigens in the joint and cause auto-immunity; nevertheless, the 'naive' T cells (called Th0) first need to become activated and acquire a pathogenic phenotype in order to be harmful. Exposure to (deranged) intestinal microbiota may be a critical factor. The aim of this review is to discuss the role of intestinal bacteria in the development of RA in the context of T cell modulation and the possible role that TLRs play in this process (Figure 1).

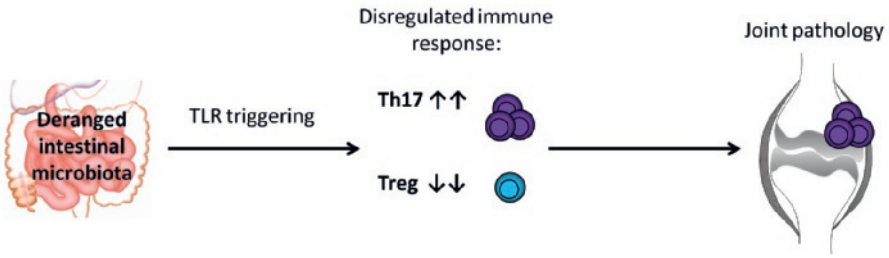

Figure 1. Exposure to deranged intestinal microbiota or a disregulated immune response to microbiota drives rheumatoid arthritis by promoting Th17 and deranging Treg cells.

Th17 cells and rheumatoid arthritis

Th17 cells protect against bacterial and fungal infections, however they also promote the development of autoimmune diseases such as multiple sclerosis, inflammatory bowel disease, psoriasis and RA [17-22]. Regulatory T cells (Tregs) downregulate inflammation and serve to prevent tissue damage and autoimmunity. The balance between Th17 cells and Tregs is strictly regulated, and imbalance is thought to promote autoimmunity [23]. In RA, increased percentages of Th17 cells have been found in peripheral blood mononuclear cells (PBMCs) of patients [22]. These Th17 cells were shown to be potent inducers of matrix metalloproteinases and proinflammatory cytokines upon interaction with synovial fibroblast, thereby contributing to joint damage [22].

Other studies found increased levels of Th17 cells and decreased levels of Tregs in peripheral blood of patients with active RA [24, 25]. Furthermore, RA patients have Tregs with decreased suppressive activity [26]. Transforming growth factor (TGF) β is a key factor involved in maintaining the Th17/Treg cell balance: TGFβ in combination with IL-6 or IL-21 promotes Th17 differentiation, but when TGFβ is present in combination with IL-2, it will induce differentiation of Tregs [27, 28]. Inhibition of IL-6 function was shown to correct the Th17/Treg cell imbalance in RA patients [24]. Targeting the Th17 pathway in autoimmune diseases such as RA is very promising [29]. However, factors promoting Th17 differentiation are poorly understood. Since specific intestinal microbiota greatly promote Th17 differentiation in intestinal mucosa, exposure to (deranged) intestinal microbiota may be a critical factor in autoimmune arthritis.

Intestinal microbiota and regulation of the immune response

Large numbers of commensal microorganisms inhabit the gastrointestinal tract of mammals. It has been shown that these commensal microbiota are essential for a proper development of the immune system, as germ-free (GF) mice possess an underdeveloped mucosal immune system [30]. GF mice have decreased numbers of lamina propria $CD4^+$ cells, hypoplastic Peyer's patches and greatly reduced immunoglobulin A (IgA) producing plasma cells [30, 31]. In addition,

also spleen and lymph nodes are underdeveloped in GF mice, as they contain poorly formed B and T cell zones [30]. Introduction of *Bacteroides fragilis* into GF mice has been shown to induce correct development of the immune system [32].

Ivanov *et al*. showed that the introduction of SFB in GF mice resulted in an increase of Th17 cells in the intestinal lamina propria [33]. In the murine gut, the presence of SFB has been shown to promote the development of Th17-mediated autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), colitis and arthritis [34-36]. Colonization of mice with *B. fragilis*, a human commensal, induces Tregs and prevents development of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis [37]. In addition, oral treatment of mice with polysaccharide A (PSA), a molecule expressed by *B.fragilis*, induced IL-10 producing Tregs and protected against EAE [38]. Another study showed that colonization of mice with microbiota belonging to the *Clostridium* species also resulted in the induction of Tregs [39]. In addition, colonization of young mice with mix of *Clostridium* species resulted in resistance to dextran sodium sulfate (DSS)- induced colitis [39]. These studies suggest that intestinal microbiota play an important role in maintaining the balance between pro- and anti-inflammatory T cells, thereby preserving intestinal homeostasis.

A recent study elegantly demonstrated the specific labeling and tracking of intestinal leukocytes [40]. It was shown that intestinal leukocytes migrate to and from the intestine at steady state [40]. In addition, the migration of intestinal Th17 cells in arthritic K/BxN mice was studied, and showed that gut derived Th17 cells end up in the spleen [40]. The fraction of gut-derived Th17 cells present in the spleen correlated with serum level of pathogenic auto antibodies [40]. This is the first study which shows that gut-derived Th17 cells can contribute to autoimmune arthritis [40].

Taken together, it is conceivable that a disturbed balance in the composition of microbiota, the so-called dysbiosis, could result in disruption of intestinal and systemic immune homeostasis. A link between intestinal microbiota and autoimmune deficiencies such as RA seems therefore plausible [41].

Rheumatoid arthritis and microbiota

Treatment with tetracycline antibiotics, in particular minocycline, was shown to significantly reduce disease activity in sero-positive RA patients with disease duration of <1 year [42]. Moreover, the commonly used DMARD sulfasalazine is known to have both anti-inflammatory and antimicrobial properties [43]. Using a small set of oligonucleotide probes detecting broad groups of bacteria, intestinal microbiota of RA patients was found different from that of fibromyalgia (FM) patients [44]. The authors did not include healthy control subjects in the study; however a group of patients with FM, having a comparable age and sex distribution and receiving similar treatment with NSAIDS drugs were included as

controls. This study showed that RA patients had significantly less *bifidobacteria* species, bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *Bacteroides fragilis* subgroup, and the *Eubacterium rectal - Clostridium coccoides* group, when compared to FM patients [44].
A recent study using 454 pyrosequencing of 16S rRNA of intestinal microbiota in stool samples found a strong correlation between the of presence of *Prevotella copri* with disease in new-onset untreated RA patients [45]. Abundance of *P. copri* in this study was inversely correlated with the presence of HLA-DRB-1 risk alleles, suggesting requirement of intestinal microbial signals in the absence of genetic predisposition factors for one to develop the disease. Another study demonstrated that fecal microbiota of RA patients contained significantly more *Lactobacilli* compared to healthy controls [46]. Altogether, the efficacy of oral antibiotic treatment and recent findings on disturbed composition of intestinal microbiota in early RA supports the involvement of microbiota in the development of RA.

Experimental evidence on the role of commensal microbiota in arthritis
The critical role of commensal microbiota in the development of arthritis has been shown in at least three spontaneous auto-immune models of arthritis. These studies showed that spontaneous disease in mice with T cell-activating genetic modifications is greatly diminished under germ-free (GF) or specified pathogen-free (SPF) conditions [13, 36, 47]. Another study showed that oral treatment with enrofloxacin, a broad-spectrum antibiotic, exacerbates collagen induced arthritis (CIA) in mice by influencing production of a number of proinflammatory cytokines such as IL-6 and IL-17 [48].
IL-1 receptor antagonist (IL-1Ra) deficient mice spontaneously develop autoimmune arthritis due to excessive IL-1 signaling [49]. Development of autoimmune arthritis in this mouse model is dependent on microbial flora, as arthritis was strongly attenuated in GF IL-1Ra$^{-/-}$ mice [13]. Colonization *with Lactobacillus bifidus* resulted in arthritis with incidence and severity scores comparable to those observed in conventionally housed mice [13]. The GF status IL-1Ra$^{-/-}$ mice resulted in a notable decrease in IL-17 and IL-1β production by splenocytes upon CD3 as well as TLR2 and TLR4 stimulation, suggesting abolishment of Th17 differentiation [13].
SKG mice have a mutation in the gene encoding an SH2 domain of ZAP-70, a signal transduction molecule in T cells. The aberrant ZAP-70 is thought to change the thresholds of T cells to thymic selection, which results in the positive selection of otherwise negatively selected autoimmunity T cells [50]. SKG mice develop chronic autoimmune arthritis under conventional conditions, however in strictly controlled SPF environment arthritis failed to develop [47]. Arthritis in SKG mice was shown to be accompanied with high sera levels of IL-6, known to be important in Th17 induction. However, in sera from SKG mice kept in SPF

conditions IL-6 was undetectable [47].
T cells of K/BxN mice express a transgenic T cell receptor which recognizes a self-peptide derive from glucose-6-phosphate isomerase (GPI). These autoreactive T cells stimulate GPI-specific B cells to produce high amounts of GPI auto-antibodies. Th17 cells seem to be driving arthritis in this model, as neutralization of IL-17 blocked the development in SPF-housed K/BxN mice [36]. Intriguingly, GF K/BxN mice have an almost complete deficiency of Th17 cells and are protected from severe arthritis [36]. Moreover, oral treatment of K/BxN mice with vancomycin or ampicillin inhibited the development of arthritis, while in neomycin-treated mice disease was aggravated [36]. Introduction of segmented filamentous bacteria (SFB), a gut-residing bacteria, in GF K/BxN mice resulted in an increase of Th17 cells in the lamina propria and in onset of arthritis [36]. These results suggest that certain intestinal microbiota are able to trigger an imbalance in the T cell response which leads to the development of autoimmune arthritis in a genetically predisposed host.

TLR-mediated interactions between bacterial antigens and the immune system
Although the mechanism by which commensal intestinal microbiota triggers the development of autoimmune diseases remains poorly understood to date, TLRs are most likely involved. TLRs recognize microbe-associated molecular patterns (MAMPs), which are shared by many microorganisms [51]. Each TLR recognizes certain MAMPs; for instance, TLR2, TLR4, TLR5 and TLR9 recognize peptidoglycans, lipopolysaccharides (LPS), flagellin, and unmethylated CpG motifs in bacterial DNA, respectively [52]. TLRs are expressed by a number of immune cells, including dendritic cells (DCs), macrophages, neutrophils, T cells and B cells, but TLRs have also been found on resident cells, such as epithelial and endothelial cells [53].
Antigen presenting cells (APCs) such as DCs and macrophages are known to express TLRs, and activation of TLRs induces the upregulation of MHC class II molecules and thereby may substantially influence the strength of the antigenic signal to T cells in the “immunological synapse” [54] (Figure 2). Furthermore, activation of TLRs induces upregulation of co-stimulatory molecules such as CD80,CD86 and CD40, which provide the second signal for T cell activation (Figure 2). The third signal for T cell activation and differentiation, the cytokine environment, is dramatically affected by the type and the extent of TLR activation (Figure 2). For instance, activation of TLR4 and TLR9 is thought to skew T cell differentiation toward the Th1 phenotype through induction of IL-12 production by DCs, whereas TLR2 activation might induce a Th2-biased immune response through production of IL-10 and IL-13 [55-61]. TLR4-induced IL-23 contributes to the expansion and survival of Th17 cells [62]. In addition, conditioned medium from TLR4-stimulated DCs or PBMCs induces Th17 differentiation and IL-17 production, a process potentiated by TGFβ [63].

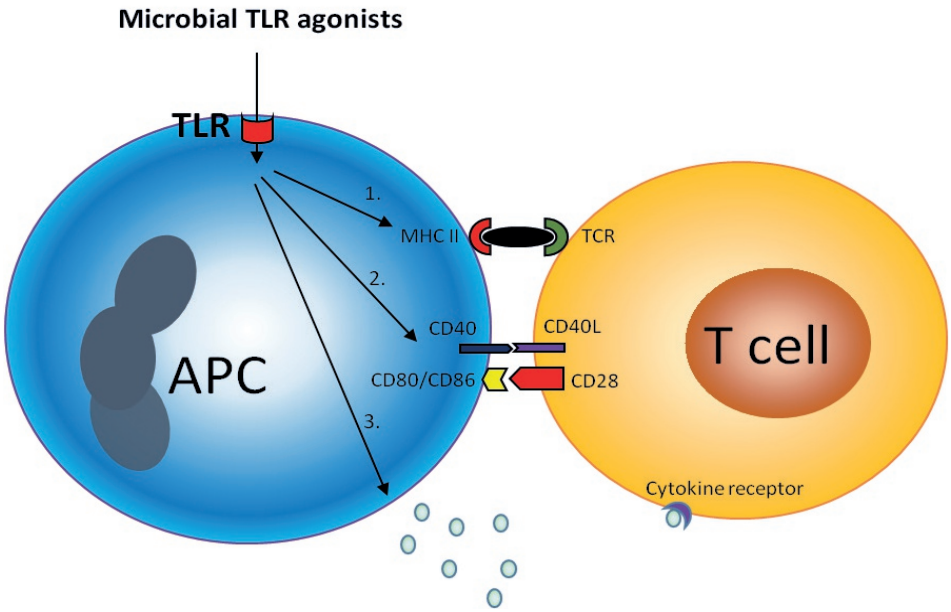

Figure 2. Toll-like receptor (TLR) activation on antigen presenting cells (APC) enhances the antigenic signal to T cells. TLR activation induces the upregulation of MHC II (1) costimulatory molecules such as CD80, CD86 and CD40 (2) and release of cytokines (3).

In addition to the type of TLR activation, the extent of TLR triggering also seems to determine the type of immune response generated. For instance, it was demonstrated that a high dose of LPS triggers a Th1 response via TLR4 while a low LPS dose results in a Th2 response to an inhaled antigen [64]. Besides APC-mediated T cell activation, some TLRs such as TLR2, 5 and 7/8 are functionally expressed on T cells and directly cause T cell activation and proliferation upon stimulation[65-67]. Others (TLR3 and 9) can enhance survival of activated CD4+ T cells [68].

Also joint resident cells are known to functionally express TLRs. RA synovial fibroblasts (RASF) for instance are known to express TLR2, TLR3, TLR4 and TLR9 [69]. Stimulation of RASF with TLR2, TLR3 and TLR4 antigens (peptidoglycans, polyinosinic:polycytidylic acid and LPS respectively) results in high production of inflammatory cytokines, MMPs and vascular endothelial growth factor and results in exacerbation of the Th1 and Th17 response [69].

A study with TLR deficient IL-1Ra$^{-/-}$ mice demonstrated that TLRs play distinct roles in the regulation of the T cell balance. In this study it was shown that Th17 differentiation is reduced in TLR4 deficient IL-1Ra$^{-/-}$ mice, while TLR2 deficiency results in a shift in T cell balance from Th2 and Treg towards Th1 cells [13]. In addition, it was shown that IL-1Ra$^{-/-}$ TLR2$^{-/-}$ mice develop a more severe arthritis compared to IL-1Ra$^{-/-}$TLR2$^{+/+}$ mice [13]. In contrast, TLR4 deficiency

in IL-1Ra$^{-/-}$ mice resulted in protection against severe arthritis [13]. This study shows that sensing of microbiota by TLRs plays a critical role in maintaining T cell balance and arthritis development.

Intestinal TLR triggering

Commensal bacteria normally do not cross the epithelial barrier. A specific population of CX3CR1 expressing cells in lamina propria has been shown to sample the lumen and interact with commensal bacteria in the lumen [70]. Although, these cells were first identified as DCs, recent studies demonstrated that CX3CR1 expressing cells in the gut are more similar to macrophages than DCs [71, 72]. This is based on the observation that CX3CR1 expressing in the intestinal lamina propria are non-migratory and cannot prime naïve T cells [71, 72]. However, another study identified CD103^{-}CD11b^{+} DCs which also express CX3CR1, these cells lacked macrophage markers such as F4/80 or CD64 [73]. CX3CR1 expressing cells were thought to be non-migratory, however a recent study showed that these cells do migrate to mesenteric lymph nodes after antibiotic-induced dysbiosis and in the absence of MyD88 [74]. Despite this finding, it is believed that the CD11b^{+} CD103^{+} classical DC subset is mainly responsible for presentation of bacterial antigen to naïve CD4^{+} T cells and Th17 differentiation in the intestinal lamina propria [74-76]. Stimulation of CD11b^{+}CD103^{+} cells with flagellin, a TLR5 ligand, resulted in the expression of high amounts of IL-23 [76]. A recent study identified a subset of CCR2-expressing CD103^{-}CD11b^{+} DCs, in lamina propria which were able to drive IL-17 production in vitro [77]. These DCs produced IL-12 and IL-23p40, and production of these cytokines increased in response to TLR4 stimulation with LPS. These DCs were found in murine as well as human lamina propria [77].

A recent study showed that luminal bacteria stimulate the recruitment of CD103^{+} DCs to the epithelium, where these DCs can also sample the lumen [78]. Recruitment of the DC to the epithelium was shown to be depended on chemokines and TLR signaling [78]. Another study showed that TLR5 is highly expressed in DCs in the intestinal mucosa, but not in splenic DCs [79]. This same study showed that TLR5$^{-/-}$ mice had increased Treg levels in the intestinal lamina propria, which suggests that TLR5 plays a role in regulating the intestinal Th17/Treg cell balance [79]. Another study demonstrated that TLR5 is expressed by CD11c^{hi} CD11b^{hi} DCs in lamina propria of mice [80]. These intestinal DCs induce the differentiation of Th1 and Th17 cells in response to flagellin [80]. In addition, TLR9 deficient mice were shown to have more Tregs and reduced Th1 and Th17 cell levels in the intestine [81].

Besides DCs also intestinal epithelial cells (IECs) in the gut are known to express TLRs. TLR 1, 2, 3, 4, 5 and 9 are known to be expressed by IECs in human small intestine, and TLR1-9 have been shown to be present on IEC in the colon [82]. In the mouse TLR1, 2, 3, 4, 5, 9 and 11 have been detected in the small intestine,

and in the colon TLR2, 3, 4 and 9 were shown to be present [82]. The expression of TLRs in the gut seems to be regulated by commensal bacteria, as it was shown that the expression of TLR2, 3, 4 and 5 was higher in colonic epithelial cells of specific pathogen-free mice when compared to GF mice [83]. An in vitro study showed that TLR4 and basolateral TLR9 stimulation on IECs drives an inflammatory response [84]. However, apical TLR9 activation resulted in the production and secretion of galectin-9, which was shown to support the development of Tregs [85].

TLR signaling on IEC is also important in maintaining the epithelial barrier, for instance TLR2 activation on epithelial cells protects against barrier disruption by upregulating the expression of zonula occludens, while TLR4 signaling results in increased intestinal permeability through up-regulation of membrane protein kinase C activity [86, 87]. Translocation of bacteria across the membrane will result in an inflammatory response in the intestinal lamina propria It has been hypothesized that intestinal barrier function, in particular the intercellular tight junctions modulated by zonulin among others, may be impaired in autoimmune disease [88, 89]. However, it is not yet clear whether this is indeed the case in individuals with autoimmune diseases such as RA.

As mentioned before a shift in the Th17/Treg cell balance is considered to be an important aspect of autoimmunity. The studies discussed here indicate an important role of intestinal TLR triggering in shaping the T helper cell subsets. This makes microbial recognition in the intestine interesting in the context of autoimmune diseases such as RA. The studies quoted here are mainly in mice. The role of intestinal TLR triggering in shaping the T cell response in humans remains mainly unclear and warrants thorough future investigation.

Specific bacteria shape the intestinal immune response.

Round *et al*. showed that polysaccharide A (PSA) of *B. fragilis* activated TLR2 directly on Tregs, which resulted in activation of these Tregs [90]. However, *B.fragilis* lacking PSA induces a Th17 response, which suggests that PSA induces an anti-inflammatory response through activation of TLR2 [90]. In addition, it was shown that PSA of *B.fragilis* prevents TNBS-induced colitis by inducing IL-10 producing Tregs. However, PSA induced protection was absent in $TLR2^{-/-}$ mice indicating that TLR2 signaling is required for PSA-induced protection [37]. Another study showed that *B. fragilis* is able to release PSA in outer membrane vesicles which are sensed by DCs through TLR2 resulting in induction of Tregs and IL-10 production [91].

A recent study showed that presentation of SFB antigens by $MHCII^{+}$ $CD11c^{+}$ intestinal DCs is required for mucosal Th17 cell differentiation [92]. In MHCII deficient mice, no SFB-induced Th17 differentiation was observed, however recovery of MHCII expression on only $CD11c^{+}$ cells was able to rescue Th17 induction [92]. In mice lacking peripheral lymph nodes and gut-associated

lymphoid tissue, SFB induced Th17 priming was unaffected, suggesting that SFB-induced T cell priming takes place in the lamina propria [92]. It is likely that the presence of SFB also triggers TLR signaling. SFB encode four types of flagellin, three of which are recognized by TLR5 [93]. In the mouse gut TLR5 is expressed by $CD11c^{hi}$ $CD11b^{hi}$ DCs in lamina propria which induce the differentiation of Th1 and Th17 cells in response to flagellin [80]. This suggests that SFB skews T cell differentiation via TLR5 triggering. Involvement of TLRs in bacteria-induced mucosal T cell responses and the subsequent systemic autoimmunity seems therefore plausible.

Conclusion

Results of multiple studies show that commensal intestinal microbiota affect the Th17 /Treg cell balance in the lamina propria and that intestinal Th17 cells can promote experimental arthritis [33, 36, 37, 39]. In addition, studies with experimental models of arthritis suggest that recognition of intestinal microbiota is required for the onset of autoimmune arthritis [13, 36, 47]. It is likely that TLRs mediate the effects of intestinal microbiota on Th cell differentiation in lamina propria. Multiple studies have shown that TLR activation plays an important role in shaping the intestinal T cell subsets [80, 84, 85, 90] In addition, the study with IL-1Ra/TLR2 and IL-1Ra/TLR4 double gene deficient mice points toward an important role of these TLRs in T cell mediated autoimmune arthritis [13]. It remained unclear how microbiota-induced Th17 cells exactly contribute to systemic autoimmunity in RA. Cross-reactivity of bacteria-specific Th17 cells to endogenous (joint-derived) antigens is a possible mechanism. Another possibility is that microbiota induced T cells promote the differentiation of self-reactive Th17 cells by changing the cytokine environment. Migration of intestinal Th17 cells to the joint and subsequent production of proinflammatory mediators is another possible mechanism. A better understanding of these yet unexplored areas and the involvement of TLR triggering by intestinal microbiota in the gut in systemic autoimmunity might offer new perspectives for manipulating the T cell response in RA patients, and may lead to the discovery of new therapeutic targets or even preventive measures.

References

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003;423(6937):356-61.
2. van der Woude D, Huizinga TW. Translating basic research into clinical rheumatology. Best practice & research Clinical rheumatology. 2008;22(2):299-310.
3. Lubberts E. Th17 cytokines and arthritis. Seminars in immunopathology. 2010;32(1):43-53.
4. Lundy SK, Sarkar S, Tesmer LA, Fox DA. Cells of the synovium in rheumatoid arthritis. T lymphocytes. Arthritis research & therapy. 2007;9(1):202.
5. Gaffen SL. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. Current rheumatology reports. 2009;11(5):365-70.
6. van den Berg WB, Miossec P. IL-17 as a future therapeutic target for rheumatoid arthritis. Nature reviews Rheumatology. 2009;5(10):549-53.
7. Klareskog L, Padyukov L, Ronnelid J, Alfredsson L. Genes, environment and immunity in the development of rheumatoid arthritis. Current opinion in immunology. 2006;18(6):650-5.
8. Andersson AK, Li C, Brennan FM. Recent developments in the immunobiology of rheumatoid arthritis. Arthritis research & therapy. 2008;10(2):204.
9. Sugiyama D, Nishimura K, Tamaki K, Tsuji G, Nakazawa T, Morinobu A, et al. Impact of smoking as a risk factor for developing rheumatoid arthritis: a meta-analysis of observational studies. Annals of the rheumatic diseases. 2010;69(1):70-81.
10. Scher JU, Ubeda C, Equinda M, Khanin R, Buischi Y, Viale A, et al. Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. Arthritis and rheumatism. 2012;64(10):3083-94.
11. de Aquino SG, Abdollahi-Roodsaz S, Koenders MI, van de Loo FA, Pruijn GJ, Marijnissen RJ, et al. Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response. Journal of immunology (Baltimore, Md : 1950). 2014;192(9):4103-11.
12. Scher JU, Abramson SB. The microbiome and rheumatoid arthritis. Nature reviews Rheumatology. 2011;7(10):569-78.
13. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. The Journal of clinical investigation. 2008;118(1):205-16.
14. Abdollahi-Roodsaz S, Koenders MI, Walgreen B, Bolscher J, Helsen MM, van den Bersselaar LA, et al. Toll-like receptor 2 controls acute immune complex-driven arthritis in mice by regulating the inhibitory Fcgamma receptor IIB. Arthritis and rheumatism. 2013;65(10):2583-93.
15. Abdollahi-Roodsaz S, van de Loo FA, van den Berg WB. Trapped in a vicious loop: Toll-like receptors sustain the spontaneous cytokine production by rheumatoid synovium. Arthritis research & therapy. 2011;13(2):105.
16. Ruyssen-Witrand A, Constantin A, Cambon-Thomsen A, Thomsen M. New insights into the genetics of immune responses in rheumatoid arthritis. Tissue antigens. 2012;80(2):105-18.
17. Matusevicius D, Kivisakk P, He B, Kostulas N, Ozenci V, Fredrikson S, et al. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. Multiple sclerosis (Houndmills, Basingstoke, England). 1999;5(2):101-4.
18. Holtta V, Klemetti P, Salo HM, Koivusalo A, Pakarinen M, Westerholm-Ormio M, et al. Interleukin-17 immunity in pediatric Crohn disease and ulcerative colitis. Journal of pediatric gastroenterology and nutrition. 2013;57(3):287-92.
19. Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, et al. Increased expression of interleukin 17 in inflammatory bowel disease. Gut. 2003;52(1):65-70.
20. Veny M, Esteller M, Ricart E, Pique JM, Panes J, Salas A. Late Crohn's disease patients present an increase in peripheral Th17 cells and cytokine production compared with early patients. Alimentary pharmacology & therapeutics. 2010;31(5):561-72.

20. Elloso MM, Gomez-Angelats M, Fourie AM. Targeting the Th17 pathway in psoriasis. Journal of leukocyte biology. 2012;92(6):1187-97.
21. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis and rheumatism. 2011;63(1):73-83.
22. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. The New England journal of medicine. 2009;361(9):888-98.
23. Samson M, Audia S, Janikashvili N, Ciudad M, Trad M, Fraszczak J, et al. Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. Arthritis and rheumatism. 2012;64(8):2499-503.
24. Wang W, Shao S, Jiao Z, Guo M, Xu H, Wang S. The Th17/Treg imbalance and cytokine environment in peripheral blood of patients with rheumatoid arthritis. Rheumatology international. 2012;32(4):887-93.
25. Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M, et al. Protein kinase C-theta mediates negative feedback on regulatory T cell function. Science (New York, NY). 2010;328(5976):372-6.
26. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunological reviews. 2006;212:60-73.
27. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007;448(7152):484-7.
28. Roeleveld DM, van Nieuwenhuijze AE, van den Berg WB, Koenders MI. The Th17 pathway as a therapeutic target in rheumatoid arthritis and other autoimmune and inflammatory disorders. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy. 2013;27(5):439-52.
29. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature reviews Immunology. 2004;4(6):478-85.
30. Macpherson AJ, Geuking MB, McCoy KD. Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology. 2005;115(2):153-62.
31. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005;122(1):107-18.
32. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485-98.
33. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proceedings of the National Academy of Sciences of the United States of America. 2011;108 Suppl 1:4615-22.
34. Stepankova R, Powrie F, Kofronova O, Kozakova H, Hudcovic T, Hrncir T, et al. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. Inflammatory bowel diseases. 2007;13(10):1202-11.
35. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
36. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(27):12204-9.
37. Ochoa-Reparaz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. Mucosal immunology. 2010;3(5):487-95.

38. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science (New York, NY). 2011;331(6015):337-41.
39. Morton AM, Sefik E, Upadhyay R, Weissleder R, Benoist C, Mathis D. Endoscopic photoconversion reveals unexpectedly broad leukocyte trafficking to and from the gut. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(18):6696-701.
40. Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? Nature reviews Immunology. 2010;10(10):735-44.
41. Stone M, Fortin PR, Pacheco-Tena C, Inman RD. Should tetracycline treatment be used more extensively for rheumatoid arthritis? Metaanalysis demonstrates clinical benefit with reduction in disease activity. The Journal of rheumatology. 2003;30(10):2112-22.
42. Hannonen P, Mottonen T, Hakola M, Oka M. Sulfasalazine in early rheumatoid arthritis. A 48-week double-blind, prospective, placebo-controlled study. Arthritis and rheumatism. 1993;36(11):1501-9.
43. Vaahtovuo J, Munukka E, Korkeamaki M, Luukkainen R, Toivanen P. Fecal microbiota in early rheumatoid arthritis. The Journal of rheumatology. 2008;35(8):1500-5.
44. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. eLife. 2013;2:e01202.
45. Liu X, Zou Q, Zeng B, Fang Y, Wei H. Analysis of fecal Lactobacillus community structure in patients with early rheumatoid arthritis. Current microbiology. 2013;67(2):170-6.
46. Hida S, Miura NN, Adachi Y, Ohno N. Cell wall beta-glucan derived from Candida albicans acts as a trigger for autoimmune arthritis in SKG mice. Biological & pharmaceutical bulletin. 2007;30(8):1589-92.
47. Doro YSI, Majewska-Szczepanik M, Marci Ska K, Szczepanik M. Partial depletion of natural gut flora by antibiotic aggravates collagen induced arthritis (CIA) in mice. Pharmacological reports : PR. 2014;66(2):250-5.
48. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. The Journal of experimental medicine. 2000;191(2):313-20.
49. Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, et al. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature. 2003;426(6965):454-60.
50. Didierlaurent A, Simonet M, Sirard JC. Innate and acquired plasticity of the intestinal immune system. Cellular and molecular life sciences : CMLS. 2005;62(12):1285-7.
51. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. International reviews of immunology. 2011;30(1):16-34.
52. Hopkins PA, Sriskandan S. Mammalian Toll-like receptors: to immunity and beyond. Clinical and experimental immunology. 2005;140(3):395-407.
53. Pasare C, Medzhitov R. Toll-like receptors and acquired immunity. Seminars in immunology. 2004;16(1):23-6.
54. Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annual review of immunology. 2002;20:709-60.
55. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. The Journal of biological chemistry. 2001;276(40):37692-9.
56. Reis e Sousa C, Hieny S, Scharton-Kersten T, Jankovic D, Charest H, Germain RN, et al. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. The Journal of experimental medicine. 1997;186(11):1819-29.
57. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nature reviews Immunology. 2003;3(2):133-46.

59. Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, et al. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. Journal of immunology (Baltimore, Md : 1950). 2003;171(10):4984-9.
60. Dillon S, Agrawal A, Van Dyke T, Landreth G, McCauley L, Koh A, et al. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. Journal of immunology (Baltimore, Md : 1950). 2004;172(8):4733-43.
61. Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, et al. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. Journal of immunology (Baltimore, Md : 1950). 2004;172(5):2739-43.
62. Kattah MG, Wong MT, Yocum MD, Utz PJ. Cytokines secreted in response to Toll-like receptor ligand stimulation modulate differentiation of human Th17 cells. Arthritis and rheumatism. 2008;58(6):1619-29.
63. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006;24(2):179-89.
64. Piggott DA, Eisenbarth SC, Xu L, Constant SL, Huleatt JW, Herrick CA, et al. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. The Journal of clinical investigation. 2005;115(2):459-67.
65. Caron G, Duluc D, Fremaux I, Jeannin P, David C, Gascan H, et al. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. Journal of immunology (Baltimore, Md : 1950). 2005;175(3):1551-7.
66. Kabelitz D. Expression and function of Toll-like receptors in T lymphocytes. Current opinion in immunology. 2007;19(1):39-45.
67. Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(9):3029-34.
68. Gelman AE, Zhang J, Choi Y, Turka LA. Toll-like receptor ligands directly promote activated CD4+ T cell survival. Journal of immunology (Baltimore, Md : 1950). 2004;172(10):6065-73.
69. Hu F, Li Y, Zheng L, Shi L, Liu H, Zhang X, et al. Toll-like receptors expressed by synovial fibroblasts perpetuate Th1 and th17 cell responses in rheumatoid arthritis. PloS one. 2014;9(6):e100266.
70. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science (New York, NY). 2005;307(5707):254-8.
71. Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O, et al. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. Mucosal immunology. 2013;6(3):498-510.
72. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. The Journal of experimental medicine. 2009;206(13):3101-14.
73. Cerovic V, Houston SA, Scott CL, Aumeunier A, Yrlid U, Mowat AM, et al. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. Mucosal immunology. 2013;6(1):104-13.
74. Diehl GE, Longman RS, Zhang JX, Breart B, Galan C, Cuesta A, et al. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. Nature. 2013;494(7435):116-20.

75. Satpathy AT, Briseno CG, Lee JS, Ng D, Manieri NA, Kc W, et al. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. Nature immunology. 2013;14(9):937-48.
76. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. Immunity. 2012;36(2):276-87.
77. Scott CL, Bain CC, Wright PB, Sichien D, Kotarsky K, Persson EK, et al. CCR2CD103 intestinal dendritic cells develop from DC-committed precursors and induce interleukin-17 production by T cells. Mucosal immunology. 2014.
78. Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, et al. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity. 2013;38(3):581-95.
79. Feng T, Cong Y, Alexander K, Elson CO. Regulation of Toll-like receptor 5 gene expression and function on mucosal dendritic cells. PloS one. 2012;7(4):e35918.
80. Uematsu S, Akira S. Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. Journal of gastroenterology. 2009;44(8):803-11.
81. Hall JA, Bouladoux N, Sun CM, Wohlfert EA, Blank RB, Zhu Q, et al. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity. 2008;29(4):637-49.
82. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature reviews Immunology. 2010;10(2):131-44.
83. Lundin A, Bok CM, Aronsson L, Bjorkholm B, Gustafsson JA, Pott S, et al. Gut flora, Toll-like receptors and nuclear receptors: a tripartite communication that tunes innate immunity in large intestine. Cellular microbiology. 2008;10(5):1093-103.
84. de Kivit S, van Hoffen E, Korthagen N, Garssen J, Willemsen LE. Apical TLR ligation of intestinal epithelial cells drives a Th1-polarized regulatory or inflammatory type effector response in vitro. Immunobiology. 2011;216(4):518-27.
85. de Kivit S, Kraneveld AD, Knippels LM, van Kooyk Y, Garssen J, Willemsen LE. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. Journal of innate immunity. 2013;5(6):625-38.
86. Li X, Wang C, Nie J, Lv D, Wang T, Xu Y. Toll-like receptor 4 increases intestinal permeability through up-regulation of membrane PKC activity in alcoholic steatohepatitis. Alcohol (Fayetteville, NY). 2013;47(6):459-65.
87. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. Gastroenterology. 2004;127(1):224-38.
88. Fasano A. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. Physiological reviews. 2011;91(1):151-75.
89. Fasano A. Leaky gut and autoimmune diseases. Clinical reviews in allergy & immunology. 2012;42(1):71-8.
90. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science (New York, NY). 2011;332(6032):974-7.
91. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis and rheumatism. 2009;60(6):1647.
92. Goto Y, Panea C, Nakato G, Cebula A, Lee C, Diez MG, et al. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. Immunity. 2014;40(4):594-607.
93. Kuwahara T, Ogura Y, Oshima K, Kurokawa K, Ooka T, Hirakawa H, et al. The lifestyle of the segmented filamentous bacterium: a non-culturable gut-associated immunostimulating microbe inferred by whole-genome sequencing. DNA research : an international journal for rapid publication of reports on genes and genomes. 2011;18(4):291-303.

Chapter 3

Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis

Chapter 3

Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis

Microbiome, 2017: 5(1): 63

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Abstract

Background

Perturbation of commensal intestinal microbiota has been associated with several autoimmune diseases. Mice deficient in interleukin-1 receptor antagonist (*Il1rn*$^{-/-}$ mice) spontaneously develop autoimmune arthritis and are susceptible to other autoimmune diseases such as psoriasis, diabetes and encephalomyelitis; however, the mechanisms of increased susceptibility to these autoimmune phenotypes are poorly understood. We investigated the role of interleukin-1 receptor antagonist (IL-1Ra) in regulation of commensal intestinal microbiota, and assessed the involvement of microbiota subsets and innate and adaptive mucosal immune responses that underlie the development of spontaneous arthritis in *Il1rn*$^{-/-}$ mice.

Results

Using high-throughput 16S rRNA gene sequencing, we show that IL-1Ra critically maintains the diversity and regulates the composition of intestinal microbiota in mice. IL-1Ra deficiency reduced the intestinal microbial diversity and richness, and caused specific taxonomic alterations characterized by overrepresented *Helicobacter* and underrepresented *Ruminococcus* and *Prevotella*. Notably, the aberrant intestinal microbiota in *IL1rn*$^{-/-}$ mice specifically potentiated IL-17 production by intestinal lamina propria (LP) lymphocytes and skewed the LP T cell balance in favor of T helper 17 (Th17) cells, an effect transferable to WT mice by fecal microbiota. Importantly, LP Th17 cell expansion and the development of spontaneous autoimmune arthritis in *IL1rn*$^{-/-}$ mice were attenuated under germ-free condition. Selective antibiotic treatment revealed that tobramycin-induced alterations of commensal intestinal microbiota, *i.e.,* reduced *Helicobacter*, *Flexispira*, *Clostridium* and *Dehalobacterium*, suppressed arthritis in *IL1rn*$^{-/-}$ mice. The arthritis phenotype in *IL1rn*$^{-/-}$ mice was previously shown to depend on Toll-like receptor 4 (TLR4). Using ablation of both IL-1Ra and TLR4, we here show that the aberrations in the *IL1rn*$^{-/-}$ microbiota are partly TLR4-dependent. We further identify a role for TLR4 activation in the intestinal lamina propria production of IL-17 and cytokines involved in Th17 differentiation preceding the onset of arthritis.

Conclusions

These findings identify a critical role for IL1Ra in maintaining the natural diversity and composition of intestinal microbiota, and suggest a role for TLR4 in mucosal Th17 cell induction associated with the development of autoimmune disease in mice.

Background

Interleukin-1 (IL-1) plays a central role in inflammation and immunity [1]. Activation of IL-1 receptor is physiologically controlled by its structural homologue and natural inhibitor, the IL-1 receptor antagonist (IL-1Ra), encoded by the *IL1rn* gene [2]. *IL1rn* knockout (*IL1rn*$^{-/-}$) mice are susceptible to a variety of autoimmune diseases including arthritis, psoriasis, diabetes and encephalomyelitis [3-7]. This indicates a critical role for IL-1Ra in protection against autoimmunity; however, the mechanisms are poorly understood.

We questioned the role of IL-1Ra in regulation of the intestinal microbiota and the involvement of mucosal immune response as an underlying mechanism for the spontaneous autoimmune arthritis in *IL1rn*$^{-/-}$ mice, which is dependent on T cells and IL-17 [4, 8]. Several studies have associated commensal microbiota with autoimmune disease in mouse models of rheumatoid arthritis (RA), diabetes and multiple sclerosis [9-13]. Importantly, the diversity and the composition of commensal intestinal microbiota are altered in patients with psoriatic and RA compared with healthy individuals [14-18]. One of the most prominent effects of microbiota is to define the balance between the pro-inflammatory CD4^{+} T helper 1 (Th1) and Th17 cells and protective regulatory T (Treg) cells, both at mucosal surfaces and systemically [19-21]. In this context, specific subsets of intestinal microbiota, such as the vancomycin-sensitive segmented filamentous bacteria (SFB), robustly induce differentiation of Th17 cells in small intestine LP (SI-LP) [22, 23]. Th17 cells are considered to play a pathogenic role in a subset of patients with RA by producing proinflammatory mediators, such as IL-17, and inducing osteoclastogenesis [24-28]. Interestingly, SFB colonization has been shown to exacerbate arthritis in K/BxN mice, an autoimmune model of arthritis arising from T cell auto-reactivity to the glycolytic enzyme glucose-6-phosphate isomerase [13, 29]. However, given that SFB were not found in human adults [30, 31], it is important to investigate the involvement of other indigenous microbiota in arthritis.

We previously described that arthritis in *IL1rn*$^{-/-}$ mice is diminished under germ-free (GF) condition [12]. We also showed that *IL1rn*$^{-/-}$ arthritis is dependent on the activation of Toll-like receptor 4 (TLR4), which affected systemic Th17 cell differentiation [12]. Here, we characterized the intestinal microbiota present in autoimmune-prone *IL1rn*$^{-/-}$ mice to clarify the nature of the microbiota that trigger arthritis and the underlying mucosal immune pathways. We also examined the role of TLR4 in the intestinal mucosal immune responses associated with arthritis.

Using high-throughput 16S rRNA gene sequencing of fecal microbiota, we demonstrate a critical role for IL-1Ra in maintaining the natural diversity and composition of commensal intestinal microbiota. We show that the aberrant *IL1rn*$^{-/-}$ microbiota increases intestinal Th17 cell differentiation, a phenotype that

that is transferable to wild-type (WT) mice by the microbiota. We also provide evidence that tobramycin-sensitive indigenous commensal intestinal bacteria contribute to arthritis in *IL1rn*$^{-/-}$ mice and identify a significant role for TLR4 in mucosal induction of IL-1β and IL-17 prior to the onset of arthritis.

Materials and methods

Mice

IL1rn$^{-/-}$ mice on a BALB/c background were kindly provided by Dr. M. Nicklin (Sheffield, UK) [32]. WT BALB/c mice were purchased from Harlan, UK. Mice were co-housed in filter-top non-individually-ventilated (non-IVC) cages in the same room in our animal facility for at least 8 weeks prior to feces collection for pyrosequencing. *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice and their *IL1rn*$^{-/-}$ *Tlr*$^{+/+}$ littermates were generated as described before [12] and used for microbiota sequencing. *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ mice were compared to non-littermate *IL1rn*$^{-/-}$ mice in this study.

Microbiota sequencing and data analysis

Fecal bacterial DNA from 15-week-old mice was isolated using phenol-, chloroform-, isoamyl alcohol-based extraction (Sigma). Sequencing was performed by DNAVision (Charleroi, Belgium) on a Roche 454 GS-FLX System using 16S rRNA gene bar-coded primers targeting the V5-V6 conserved DNA regions (forward primer 784F: 5'-AGGATTAGATACCCTGGTA-3', reverse primer 1061R: 5'-CRRCACGAGCTGACGAC-3') [33]. For gene sequence analysis, a customized workflow based on Quantitative Insights Into Microbial Ecology (QIIME version 1.2) was adopted (http://qiime.org/) [34]. Settings recommended in QIIME 1.2 tutorial were applied. Additionally, reads were filtered for chimeric sequences using Chimera Slayer as described before [35]. OTU clustering was performed with settings as recommended by QIIME [36] using an identity threshold of 97%. The Ribosomal Database Project classifier version 2.2 was used for taxonomic classification [37]. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. For statistical analysis and generation of figures, a custom QIIME implemented R-package, SciPy [38] (www.Scipy.org), Graphpad Prism version 5.0, and Microsoft® Office Excel® 2007 were adopted. Presence of SFB was assessed by real-time quantitative PCR (qPCR) on fecal DNA using SFB specific primers as described before [39]. The delta Ct (cycle threshold) value was calculated for SFB-specific rRNA gene relative to the total (conserved) bacterial 16S rRNA genes amplified using universal bacterial primers to correct for the total bacterial DNA input. Data are presented as delta Ct (ΔCt) and relative SFB expression calculated as $2^{-\Delta Ct}$ x 10,000 (Supplementary Table 3).

Microbiota transfer and co-housing

IL1rn$^{-/-}$ microbiota were transferred to WT mice by oral gavage of 200 µl of a homogenized *IL1rn*$^{-/-}$ fecal suspension prepared in sterile PBS. Immediately hereafter, the gavaged WT mice were co-housed with *IL1rn*$^{-/-}$ mice in the same individually-ventilated cage for a period of 6 weeks to ensure sustained microbiota transfer by coprophagy. The control WT mice were gavaged with their own fecal suspension and housed separate from *IL1rn*$^{-/-}$ fecal-transplanted mice in IVC cages. To verify microbiota of conventional *IL1rn*$^{-/-}$ mice can trigger arthritis, GF *IL1rn*$^{-/-}$ mice received either 200 µl of sterile water or 200 µl fecal suspensions of conventional *IL1rn*$^{-/-}$ mice and were monitored for the development of arthritis for 8 weeks. In some studies, *IL1rn*$^{-/-}$ mice were co-housed with *IL1rn*$^{-/-}$*Tlr4*$^{-/-}$ mice for 10 days before analysis of LP T cells.

Antibiotic Treatments and Reconstitution with SFB

Intestinal microbiota were depleted using a cocktail of metronidazole (Acros Organics), neomycin trisulfate (Sigma) and ampicillin sodium salt (Sigma) (all 1 g/l) provided in drinking water for 1 week. Indicated groups received 200 µl fecal suspensions of SFB-monocolonized mice by oral gavage 1 week after ceasing antibiotics. For single antibiotic treatments, ampicillin sodium salt (1 g/l), metronidazole (1g/l), vancomycin hydrochloride (0.5 g/l, Fisher Scientific) or tobramycin sulfate (1 g/l, Centrafarm) was added to drinking water for 8 weeks and refreshed once a week. Sucrose (6 g/l) was added to drinking water of all groups including controls during treatments.

Isolation of Lamina Propria Cells

Lamina propria mononuclear cells were isolated from small intestine and colon after removing mesenteric fat and Peyer's patches, followed by incubation with 33 mM EDTA on ice for 30 minutes to remove epithelial cells, and subsequent digestion with 1 mg/ml collagenase-D (Roche) and 10 µg/ml DNAse I (Sigma) at 37 °C for three cycles of 15 minutes. LP lymphocytes were then harvested at the interphase of a 40:80% percoll gradient (Sigma), washed thoroughly and used in culture as described below.

Cell Cultures and Cytokine Measurements

SI-LP mononuclear cells (4 x 10^5 cells/well), LN cells (2 x 10^5 cells/well) and splenocytes (1 x 10^5 cells/well) were cultured in round-bottom 96 well plates in the presence of PMA (50 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) for 5 or 6 hours, as indicated in Figure Legends. SI-LP cells were also cultured for 24 hours in the presence of *IL1rn*$^{-/-}$ or *IL1rn*$^{-/-}$*Tlr4*$^{-/-}$ complete microbial antigens (1:200 v/v ratio) prepared by autoclaving the *IL1rn*$^{-/-}$ fecal pellets dissolved in PBS, and then centrifuging the suspension at 2000 rpm for 5 minutes. Cytokine levels in culture supernatants were measured by Luminex using the mouse cytokine/chemokine

magnetic bead kit (Milliplex and Bio-Rad).

Flow Cytometry
For intracellular cytokine staining, SI-LP cells were incubated with PMA (50 ng/ml; Sigma), ionomycin (1 µg/ml; Sigma), and Brefeldin A (1 µl/ml; BD Biosciences) at 37 °C for 4 hours. Cells were stained with fixable viability dye Efluor780 (eBioscience), anti-TCRβ-FITC (Biolegend) or anti-CD3-PE (BD pharmigen), and anti-CD4-APC (Biolegend), then fixed and permeabilized using fixation/permeabilization buffer (eBioscience) and stained with anti-IL-17-FITC (Biolegend), IL-17-PECy7 (Biolegend) or anti-IFNγ-FITC (BD pharmingen) in permeabilization buffer (eBioscience).

Assessment of Arthritis.
Severity of arthritis was scored using a previously standardized arbitrary scoring system on a 0-2 scale per paw [12]. Arthritis developed only in ankle joints (maximum score of 4). For histology, total ankle joints were isolated and fixed in 4% formaldehyde for 4 days, thereafter decalcified in 5% formic acid and embedded in paraffin. Tissue sections of 7µm were stained using Haematoxylin & Eosin to study synovial inflammation, cartilage destruction and bone erosion. Each parameter was scored on a scale from 0-3 in a blinded manner.

Statistics
Group measures are expressed as mean + SEM. Statistical significance was tested using an unpaired two-tailed Mann-Whitney U test to compare two and ANOVA to compare more groups, with Bonferroni correction for multiple testing when applicable (GraphPad Prism 5.0). Arthritis scores were compared using repeated measures ANOVA with Bonferroni correction. Significance is indicated on figures as follows: n.s. (not significant), *P < 0.05, **P < 0.01, ***P < 0.001.

Results

IL-1Ra maintains the biodiversity and richness of commensal intestinal microbiota

To identify intestinal microbiota associated with arthritis, we sequenced fecal bacterial 16S rRNA genes of *IL1rn*$^{-/-}$ and age- and gender-matched WT control mice. Fecal microbiota were analyzed as an unselected representation of the overall microbial communities in the intestines. Considering differential roles of TLR2 and TLR4 in *IL1rn*$^{-/-}$ arthritis [12], we sequenced samples of *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice in parallel. The average sequencing depth and total numbers of reads and operational taxonomic units (OTU) per experimental group as well as the hierarchical weighed UniFrac cluster analysis at the genus level are shown in Supplementary Table 1 and Supplementary Figure 1.

Principal coordinates analysis (PCoA) based on an unweighted UniFrac analysis of intestinal microbiota showed that *IL1rn*$^{-/-}$ microbiota is profoundly different from the WT microbiota (Figure 1A). WT and *Il1rn*$^{-/-}$ mice formed clear, separate clusters regardless of the cage or litter of origin (Figure 1A). Strikingly, microbial composition of *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ mice were indistinguishable, while *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice formed another distinct cluster (Figure 1A). To assess the effects of familial transmission and lineage origin versus the effect of the genotype (WT or *IL1rn*$^{-/-}$), we compared the UniFrac distances within a litter with UniFrac distances across litters of the same genotype as well as the opposite genotype, similar to the study by Ubeda *et al*. This analysis showed that the effect of the lineage origin and litter was limited in our experimental setting, because, as long as the genotype remained the same, the UniFrac distances across different litters were very similar to the UniFrac distances within the litters (Supplementary Figure 2A). This was true for both WT and *Il1rn*$^{-/-}$ groups. Importantly, the UniFrac distances were significantly higher when mice from different genotypes were compared, indicating a higher level of dissimilarity (Supplementary Figure 2B). Therefore, the effect of the genotype (*Il1rn*-deficiency) on the overall microbiota composition was significantly higher than any litter and cage effect. In addition, *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ mice showed significantly reduced number of OTUs and loss of microbial diversity based on the Shannon index, the rarefaction curves of phylogenetic distance (PD) whole tree, and the diversity index bootstrapped for the number of retrieved sequences (Figure 1B-E). IL-1Ra deficiency also resulted in loss of species richness estimated by Chao index (Figure 1F). These effects were fully or partially restored in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice (Figure 1B-F). Altogether, these data strongly suggest that IL-1Ra plays a critical role in maintaining the intestinal microbial diversity, and that the loss of diversity in *IL1rn*$^{-/-}$ mice partially depends on TLR4.

Specific taxonomic alterations characterize the dysregulated microbiota of autoimmune-prone IL1rn$^{-/-}$ mice

The phylogenetic tree in Figure 2 summarizes the observed alterations in relative abundances of microbial taxa. Compared with WT microbiota, we found a highly significant overrepresentation of the genus *Helicobacter* (P = 0.004, Bonferroni corrected), and a significant underrepresentation of the genus *Prevotella* (P = 0.008, Bonferroni corrected) (Figure 2 and Supplementary Table 2). In addition, *IL1rn*$^{-/-}$ intestinal microbial composition was characterized by expansion of *Butyricimonas*, *Rikenella* and *Streptococcus* by 10, 3.7 and 2.4 folds (P = 0.0048, P = 0.0022 and P = 0.0032, respectively, Bonferroni uncorrected), along with a decrease in *Parasutterella*, *Xylanibacter*, *Ruminococcus* and *Barnesiella* by 10, 6.9, 2.7 and 1.4 folds (P = 0.040, P = 0.0004, P = 0.0099 and P = 0.0005, respectively, Bonferroni uncorrected), respectively (Figure 2 and Supplementary Table 2). Notably, we were unable to identify any OTUs in our dataset that could

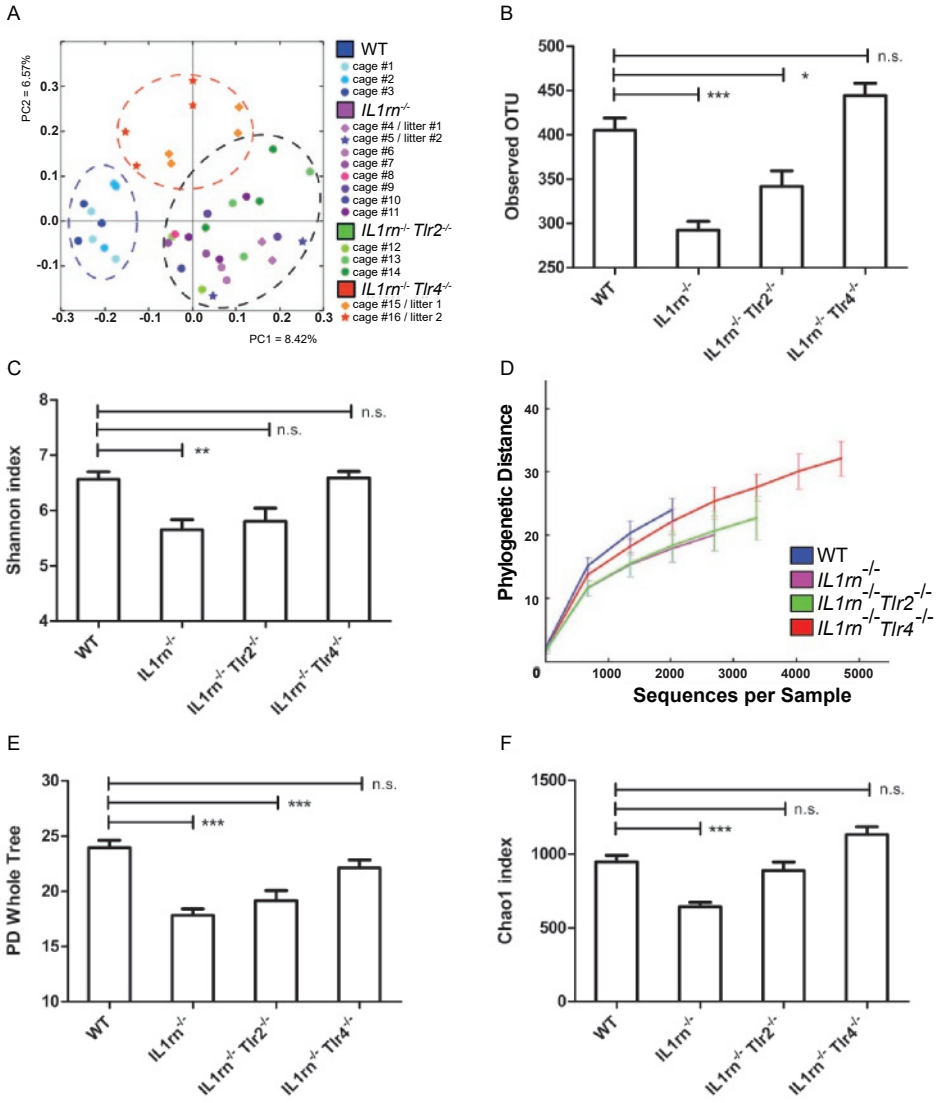


Figure 1. IL-1Ra deficiency skews intestinal microbial composition and reduces its diversity in a TLR4-dependent manner. (A) Principal coordinates analysis (PCoA) based on an unweighted UniFrac analysis of the intestinal microbial composition where samples of mice from different cages and litters are highlighted with different colors. The position and distance of data points indicates the degree of similarity in terms of both presence and relative abundance of bacterial taxonomies. (B) Number of observed operational taxonomic units (OTUs), (C) Shannon index of microbial diversity, (D) alpha diversity rarefaction curves of phylogenetic distance (PD) whole tree, (E) PD whole tree, bootstrapped for 2000 reads per sample, averaged of 4 trials, (F) and Chao index are shown. Data (mean + SEM) represent 16S rRNA gene 454-pyrosequencing analysis of intestinal microbiota of WT (n=9), *IL1rn*$^{-/-}$ (n=15), *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ (n=8) and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ (n=8) mice. n.s. = not significant, *P ≤ 0.05 and ***P ≤ 0.001, by Mann-Whitney U test. See also Supplementary Figure 1 and Supplementary Table 1.

be classified as SFB (family *Clostridiaceae*, genus *Candidatus Arthromitus*). Moreover, none of the 27 present OTUs assigned to the family *Clostridiaceae* aligned with the known SFB 16S gene sequences in The Ribosomal Database Project [30]. However, SFB were detectable by qPCR in fecal samples of all WT mice and most of the *Il1rn*$^{-/-}$ mice (Supplementary Table 3). Although WT mice tended to have slightly more SFB, the level of SFB colonization was not significantly different between the groups (Supplementary Table 3).
Altogether, these data suggest that multiple yet specific microbial taxa are regulated by the physiologic expression of IL-1Ra. Therefore, a complex set of aberrant microbiota may affect the (mucosal) immune response and contribute to the autoimmune disease in *IL1rn*$^{-/-}$ mice.

IL1rn$^{-/-}$ intestinal microbiota potentiate IL-17 production by intestinal lamina propria lymphocytes

To assess the effect of IL-1Ra deficiency on the mucosal T cell response, we cultured enzymatically isolated lamina propria lymphocytes (LPL) *ex vivo* in the presence of PMA and ionomycin. The production of the Th1 signature cytokine IFNγ was low and not altered by the IL-1Ra deficiency (Figure 3A, gating strategy shown in Supplementary Figure 3); however, we observed a marked increase in the production of IL-17 by *IL1rn*$^{-/-}$ LPLs compared with WT LPLs (Figure 3B). Flow cytometry analysis of lamina propria cells of WT and *IL1rn*$^{-/-}$ mice verified a significant, clear increase of IL-17-producing TCRβ^{+} CD4^{+} cells in *IL1rn*$^{-/-}$ mice, while TCRβ^{-} cells in LP produced similar amounts of IL-17 in WT and *IL1rn*$^{-/-}$ mice (Supplementary Figure 4). This suggests that Th17 cells, not γδT cells, are the source of increased IL-17 production in LP of *IL1rn*$^{-/-}$ mice. Production of IL-4, IL-6 and TNFα was not affected (Figure 3C and data not shown). Interestingly, the production of IL-17 but not IFNγ by lymphocytes in joint-draining lymph nodes (dLN) was significantly increased in *IL1rn*$^{-/-}$ mice compared with WT mice (Figure 3D and E). This was paralleled by a concomitant decrease in the production of the Th2-related cytokine IL-4 in *IL1rn*$^{-/-}$ mice (Figure 3F).
To identify a potential causative relationship between the aberrant microbiota and enhanced mucosal IL-17 production, we transferred *IL1rn*$^{-/-}$ microbiota to WT mice by oral gavage followed by immediate co-housing of the two mouse strains for up to 6 weeks. Transfer of *IL1rn*$^{-/-}$ microbiota clearly potentiated IL-17 production by SI-LP T cells in WT recipients as early as 10 days post fecal transfer and co-housing, without affecting IFNγ and IL-4 (Figure 3G-I). This indicated that *IL1rn*$^{-/-}$ intestinal microbiota causes a shift in the LP T cell balance in favor of Th17 cells. However, this was not sufficient for the development of arthritis in WT animals during the 6 weeks follow-up period. This suggests that additional (genetic) susceptibility of the host, as in *IL1rn*$^{-/-}$ mice, is required for the development of arthritis. Furthermore, co-housing with WT mice did not affect the development of arthritis in *IL1rn*$^{-/-}$ mice (not shown).

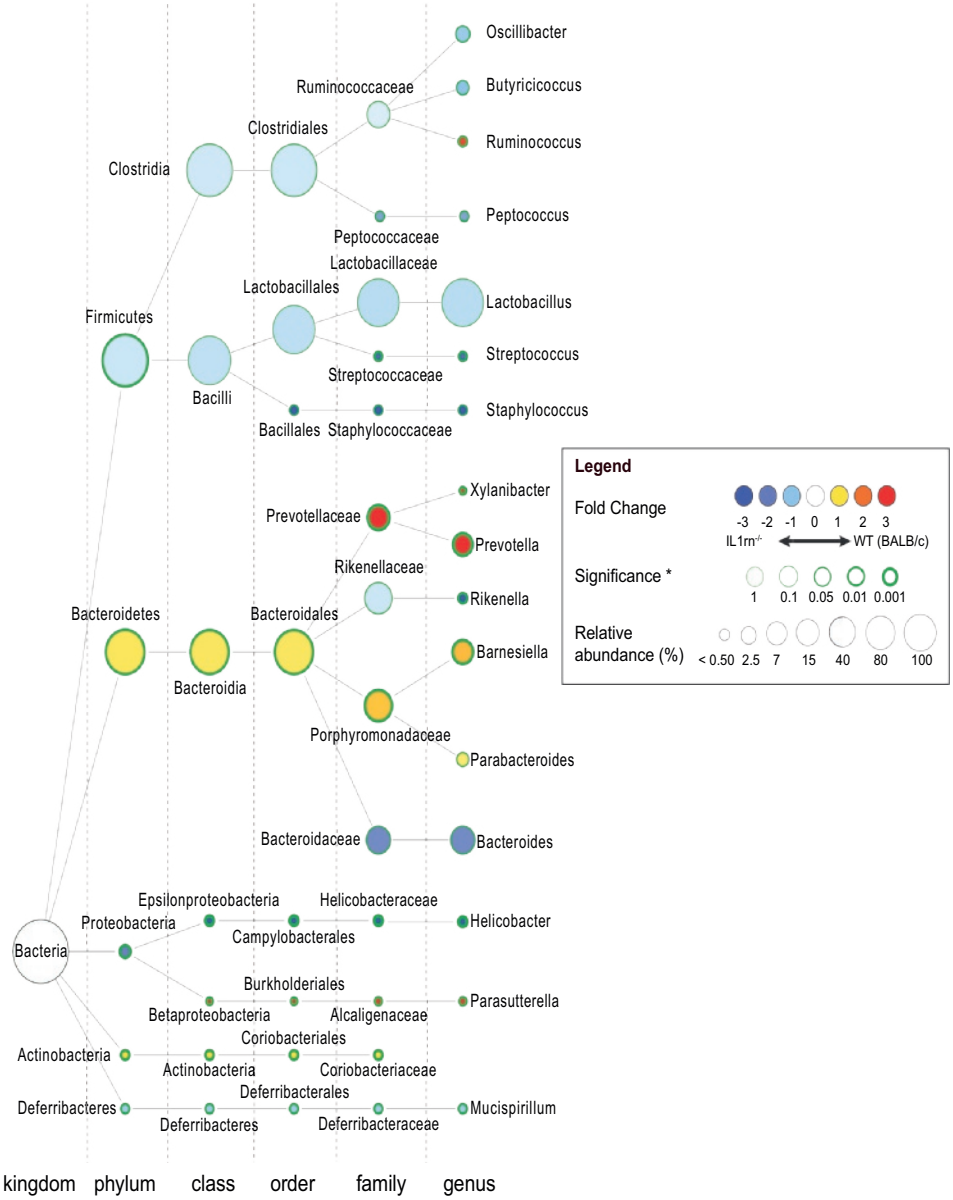


Figure 2. IL-1 receptor antagonist controls relative abundance of specific intestinal microbial taxa. Phylogenetic tree created by Cytoscape software showing specific changes in intestinal microbial community at different taxonomic levels induced by IL-1Ra deficiency. Nodes represent taxa, and the size of each node represents its relative abundance. The color red indicates a decrease and blue represents an increase of relative abundance in *IL1rn*$^{-/-}$ compared with WT mice. The thickness of the green border indicates the degree of the statistical significance by Mann-Whitney U test. See also Supplementary Table 2.

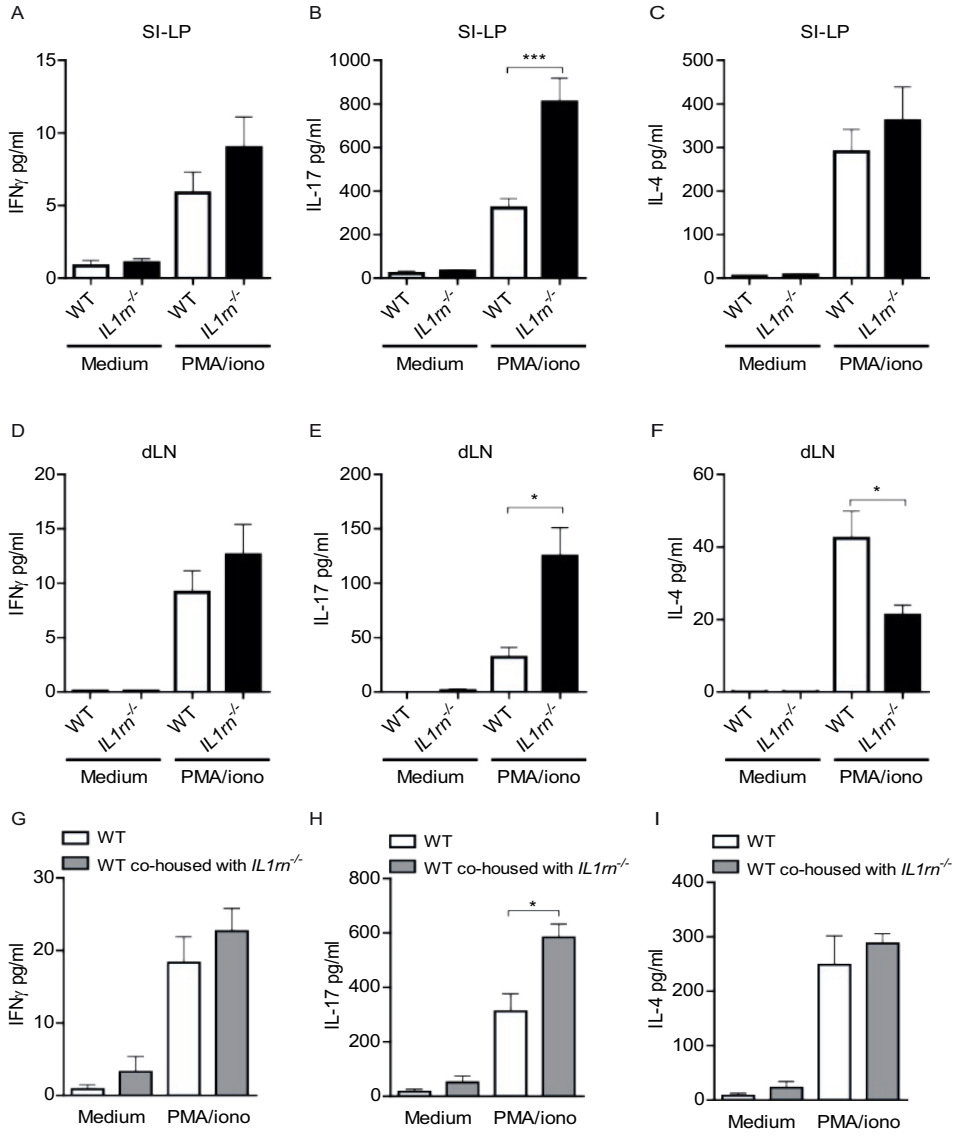


Figure 3. *Il1rn*$^{-/-}$ intestinal microbiota potentiate IL-17 production in intestinal lamina propria and joint-draining lymph nodes. (A-I) Production of prototypic Th1, Th17 and Th2 cell cytokines (IFNγ, IL-17A and IL-4, respectively) by SI-LP (A-C and G-I) and draining lymph node (dLN) lymphocytes (D-F). Cells were isolated from WT and *IL1rn*$^{-/-}$ mice (A-F), or WT mice transplanted with *IL1rn*$^{-/-}$ feces and co-housed with *IL1rn*$^{-/-}$ mice for 10 days (G-I). Cells were stimulated *ex vivo* with PMA and ionomycin in duplicates for 5 hours, and cytokines were measured by Luminex assay. Data represent mean + SEM of a representative experiment with n=5 (A-C) and n=3 (D-I) mice per group, each stimulated in duplicate. n.s. = not significant, *P ≤ 0.05 and ***P ≤ 0.001, by Mann-Whitney U test. See also Supplementary Figure 2.

Potentiated Th17 response and spontaneous arthritis in IL1rn$^{-/-}$ mice highly depend on the presence of commensal microbiota

To determine whether the increase in intestinal Th17 cells and spontaneous arthritis in *IL1rn*$^{-/-}$ mice depends on commensal microbiota, we established Germ-free (GF) *IL1rn*$^{-/-}$ mice. Flow cytometry analysis of LPLs showed that germ-free condition had no significant effect on the percentage of Th1 cells while reducing the numbers of Th1 cells in SI-LP (Figure 4A and B). In contrast, both the percentage and the number of SI-LP Th17 cells were substantially reduced in GF compared with conventional (CV) mice (Figure 4C and D). This strongly suggests that the skewed intestinal T cell balance in *IL1rn*$^{-/-}$ mice is largely microbiota-dependent.

In agreement with IL-17-dependence of *IL1rn*$^{-/-}$ arthritis [4, 8] and in line with our previous observations [12], GF *IL1rn*$^{-/-}$ mice showed a clear sustained protection from arthritis with on average three weeks delay in disease onset (Figure 4E). In addition, transfer of conventional *IL1rn*$^{-/-}$ microbiota to GF *IL1rn*$^{-/-}$ mice re-induced arthritis and resulted in a severe disease comparable to that in conventional *IL1rn*$^{-/-}$ mice (Supplementary Figure 5). Therefore, *IL1rn*$^{-/-}$ commensal microbiota, although not sufficient to induce arthritis in a WT host, are critical for the full development of arthritis in *IL1rn*$^{-/-}$ mice. Consistently, we also observed a robust reduction of IL-17, but not IFNγ, production in spleen and most notably in joint-adjacent lymph nodes of GF mice (Figure 4F and G). These effects were accompanied by a significant reciprocal increase in Th2-related cytokines IL-4 and IL-10 as well as IL-2 in spleens of GF mice (Figure 4H and Supplementary Figure 6). These data support modulation of extra-intestinal immune response by intestinal microbiota during arthritis.

Tobramycin-induced alteration of intestinal microbiota suppresses arthritis in IL1rn$^{-/-}$ mice

The lack of microbiota in GF mice is not limited to the intestines. To determine whether intestinal microbiota serve as a relevant trigger for arthritis, we first depleted intestinal microbiota in conventionally-housed mice using a cocktail of metronidazole, neomycin and ampicillin. Treatment of 5-week-old *IL1rn*$^{-/-}$ mice for only 1 week suppressed arthritis over a sustained period, i.e. 6 weeks after ceasing antibiotics (Figure 5A). This indicated that abrogation of arthritis in GF mice (Figure 4C) is not due to an immature immune system and, more importantly, can be reproduced by the sole eradication of intestinal microbiota. Interestingly, colonization of the antibiotic-treated mice with SFB as model organisms inducing SI-LP Th17 cells was sufficient to fully restore arthritis (Figure 5A).

To determine which subset of *IL1rn*$^{-/-}$ microbiota triggers arthritis, we first compared the effects of treatment with ampicillin, broadly targeting aerobic bacteria, and metronidazole, broadly targeting anaerobic bacteria. To our suprise

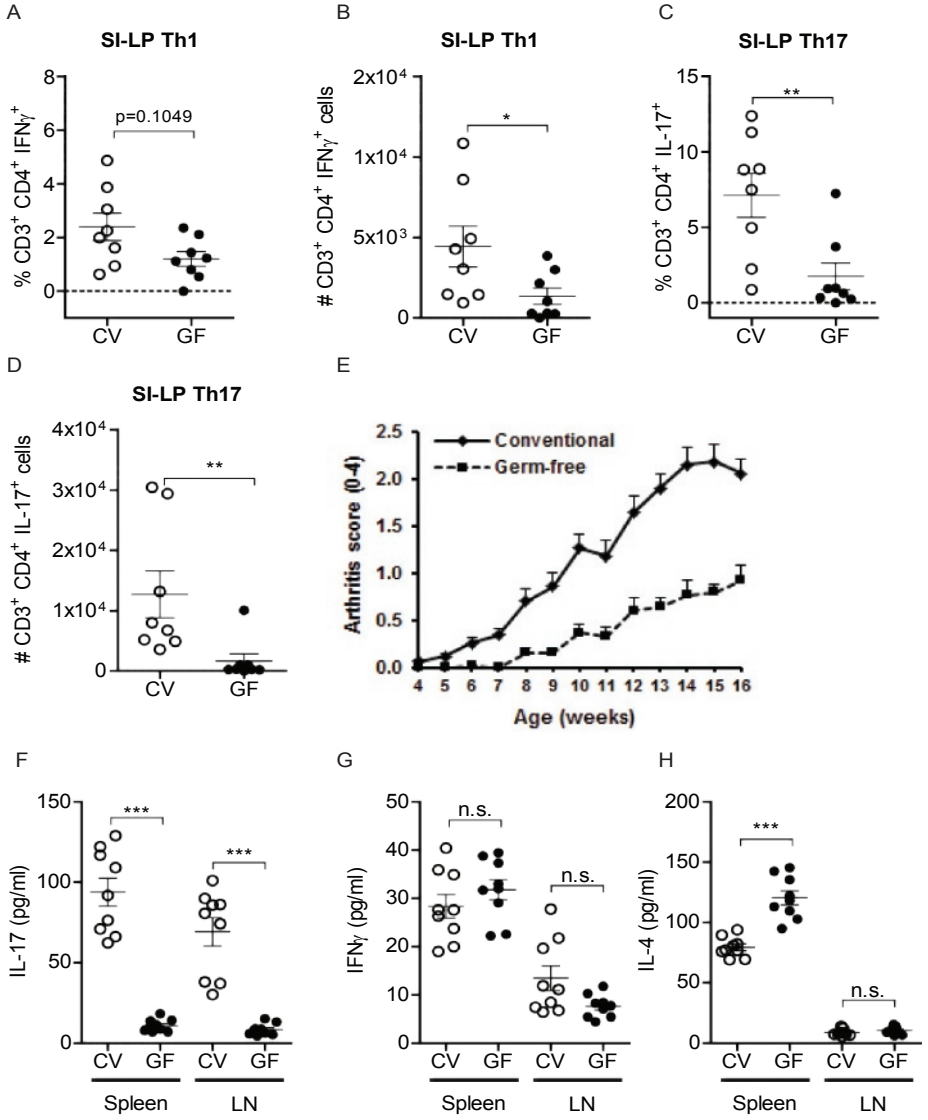


Figure 4. Commensal microbiota drive potentiated Th17 response and spontaneous arthritis in *IL1rn*$^{-/-}$ mice. (A-D) Frequency and numbers of IFNγ-producing (A,B) and IL-17-producing (C,D) CD3^{+}CD4^{+} SI-LP cells. Data are pooled from three independent experiments. (E) Arthritis severity scores of conventional (CV, n=12) and germ-free (GF, n=11) *IL1rn*$^{-/-}$ mice of a representative experiment. Scale 0-2 for each hind paw. Mean + SEM is shown. (F-H) Production of IFNγ, IL-17 and IL-4 upon *ex vivo* stimulation of spleen and lymph node cells from CV and GF mice with PMA and ionomycin for 6 hours, as measured by Luminex assay. n=3 mice per group of each stimulated in triplicate. n.s. = not significant, **P ≤ 0.01 and ***P ≤ 0.001, by Mann-Whitney U test. See Supplementary Figure 3 for gating strategy.

only metronidazole showed efficacy in reducing arthritis severity (Figure 5B). This suggests involvement of anaerobic bacteria in the progression of arthritis. We next compared the effects of more selective antibiotics tobramycin and vancomycin, the latter of which has been reported to eradicate SFB and inhibit SFB-induced lamina propria Th17 cells and arthritis [13, 22, 40]. These experiments revealed that although SFB were able to exacerbate arthritis in *IL1rn*$^{-/-}$ mice (Figure 5A), only tobramycin but not vancomycin significantly diminished arthritis (Figure 5C). To understand the changes in the microbiota induced by tobramycin treatment, we compared 16S rRNA gene sequences of fecal microbiota at the end-point of tobramycin treatment with the baseline microbiota. Among taxa with >0.1% relative abundance, tobramycin treatment resulted in a near-complete elimination of the genera *Helicobacter* and *Flexispira* (both belonging to the family Helicobacteraceae). In addition, a strong and highly significant reduction in the genera *Clostridium* and *Dehalobacterium* was observed (Supplementary Figure 7 and Supplementary Table 4). Other changes in the microbiota did not reach the statistical significance after Bonferroni correction for multiple testing. Therefore, tobramycin-induced alterations in these indigenous *IL1rn*$^{-/-}$ microbiota taxa resulted in suppression of arthritis. This was confirmed by histological examination of arthritic joints which showed a significant reduction of synovial inflammation as well as cartilage destruction and a non-significant reduction in bone erosion upon treatment with tobramycin (Figure 5D and E).

Aberrations of the intestinal microbiota and LP IL-17 production in IL1rn$^{-/-}$ mice partly depend on TLR4.

TLR4 plays a major role in recognition of Gram-negative bacteria [41]. We previously showed that *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice have a marked and sustained reduction of arthritis [12].Therefore, we assessed whether TLR4 plays a role in alterations of the intestinal microbiota and the induction of LP Th17 cells. A detailed analysis of the intestinal microbiota showed that in addition to the TLR4-dependent loss of microbial diversity in IL-1Ra-deficient mice (Figure 1B-F), alterations in *Ruminococcus*, *Streptococcus* and *Xylanibacter* were partially dependent on TLR4 and were restored in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice (Figure 6A). Abundance of *Prevotella* was also restored to a statistically significant, yet minor extent (Figure 6B). In total, 11 out of 44 taxa significantly altered in *IL1rn*$^{-/-}$ mice were normalized toward the WT levels in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice (Supplementary Table 2).

We next examined the role of TLR4 in the mucosal T cell response in *IL1rn*$^{-/-}$ mice. Th17 cells require transforming growth factor-β and IL-6, plus IL-1β in mouse, for initial differentiation, and IL-23 for their functional maturation and pathogenic function [24, 42]. To determine the role of TLR4 in response to *IL1rn*$^{-/-}$ intestinal microbial antigens, we cultured SI-LP mononuclear cells from *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice *ex vivo* with autoclaved *IL1rn*$^{-/-}$ intestinal microbiota.

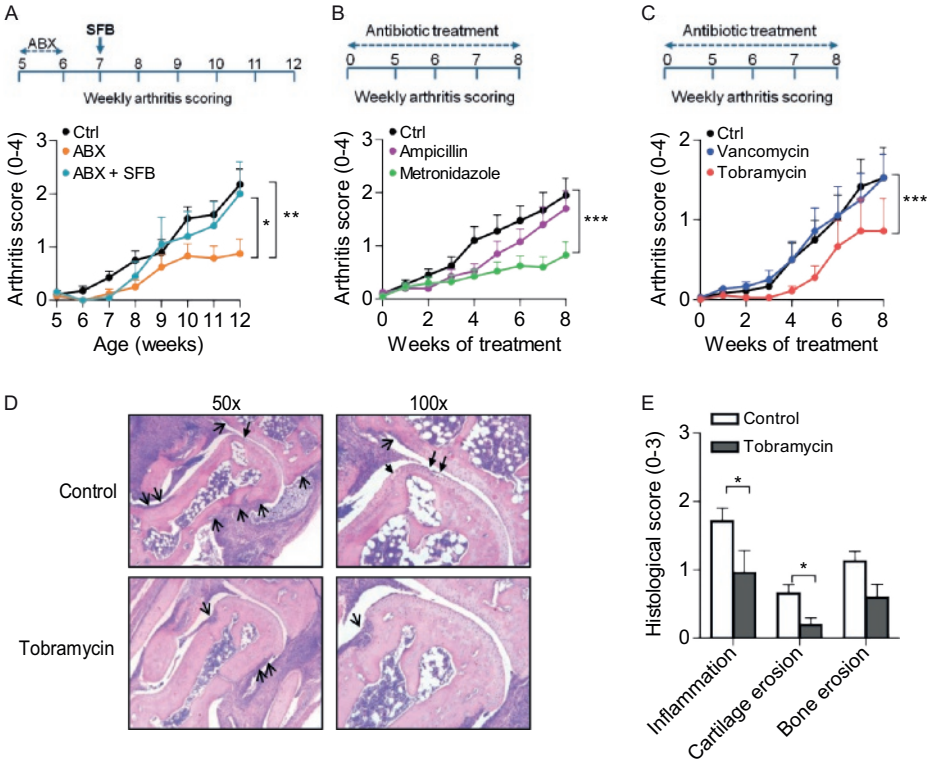


Figure 5. Commensal intestinal anaerobic tobramycin-sensitive microbiota promote arthritis in *IL1rn*$^{-/-}$ mice. (A) Arthritis severity scores (0-2 per paw) of *IL1rn*$^{-/-}$ mice treated with a cocktail of metronidazole, neomycin and ampicillin (ABX) for 7 days (week 5 to 6), followed by re-colonization with SFB (ABX + SFB) one week after ending ABX treatment (at week 7). (B-C) Arthritis severity scores of *IL1rn*$^{-/-}$ mice either untreated (Ctrl) or treated with the mentioned antibiotic for 8 weeks. Data show mean + SEM of n=5-7(A) and n=10 (B-C) mice per group. *P ≤ 0.05, ***P ≤ 0.001, by repeated measures ANOVA with Bonferroni correction for multiple testing. (D) Representative images of ankle joint sections of control and tobramycin-treated mice stained with hematoxylin and eosin illustrating decreased synovial inflammation, cartilage destruction (closed arrows) and bone erosion (open arrows). Original magnification x50 (left panels) and x100 (right panels). (E) Histopathologic scores (mean + SEM) of synovial inflammation, cartilage destruction and bone erosion in control and tobramycin-treated *IL1rn*$^{-/-}$ mice. n=9 per group. *P ≤ 0.05, by Mann-Whitney U test.

SI-LP mononuclear cells from *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice produced significantly less IL-1β (Figure 7A, P = 0.0042). Furthermore, the induction of IL-23 and IL-6 by *IL1rn*$^{-/-}$ fecal microbiota was partly TLR4-dependent (Figure 7B and C, P = 0.0014 and P = 0.009, respectively). Reduced cytokine production in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice was not due to an altered composition of mononuclear cells in the SI-LP, because the percentage and abundance of CD11c^{+} MHCII^{+} DCs as well as distinct subsets of CD103^{+} CD11b^{+}, CD103^{+} CD11b^{-} and CD11b^{+} CD103^{-} phagocytes were similar between *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice (data not shown).

Importantly, stimulation of *IL1rn*$^{-/-}$ LP mononuclear cells with *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ fecal microbial antigens induced similar concentrations of IL-1β, IL-23 and IL-6 (Supplementary Figure 8). This suggests that the altered composition of microbiota in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice as such is not responsible for the lower production of these cytokines. These observations imply a significant role for TLR4 in intestinal production of the cytokines involved in LP Th17 differentiation in *IL1rn*$^{-/-}$ mice.

When cultured *ex vivo* with PMA and ionomycin, SI-LP cells from *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice produced significantly less IL-17 compared with cells from *IL1rn*$^{-/-}$ *Tlr4*$^{+/+}$ mice before the onset of arthritis (P = 0.0028; Figure 7D). The amount of IFNγ produced in this culture was about 50 folds lower than IL-17 and was significantly reduced in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice as well (Figure 7E). However, IL-4 levels remained unaffected (Figure 7F). LP T cells from *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice still produced significantly less IL-17 and IFNγ when these mice were co-housed with *IL1rn*$^{-/-}$ (*Tlr4*$^{+/+}$) mice to transfer the microbiota (Figure 7G-I).

Therefore, reduced LP IL-17 production in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice is a result of the difference in host TLR4 expression rather than altered microbiota in *IL1rn*$^{-/-}$*Tlr4*$^{-/-}$ versus *IL1rn*$^{-/-}$ mice. Stimulation of LP mononuclear cells of these co-housed mice with fecal microbial antigens confirmed that cells from *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice produce lower amounts of IL-1β, IL-23 and IL-6 regardless of stimulation with *IL1rn*$^{-/-}$ or *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ microbiota (Supplementary Figure 9). These data suggest that TLR4 activation contributes to intestinal LP production of IFNγ and most notably IL-17, and these effects precede the onset of arthritis.

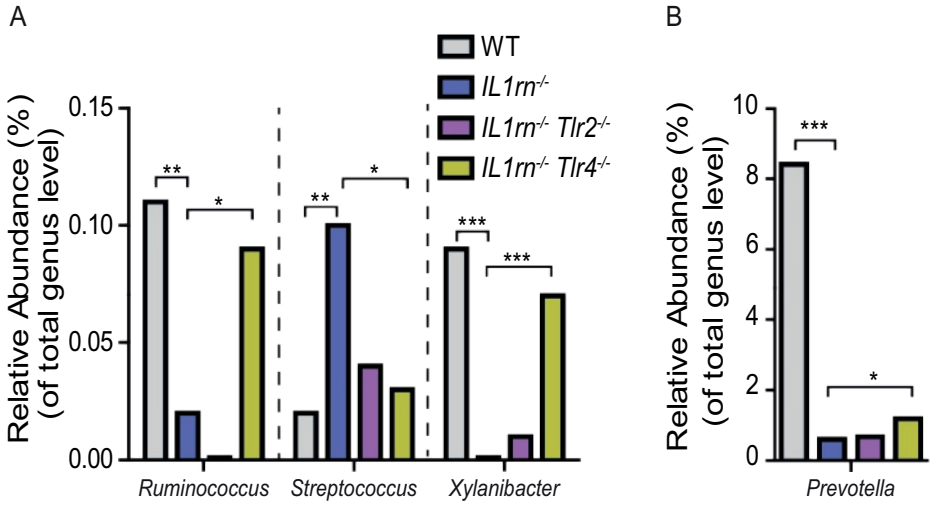


Figure 6. Alteration of specific intestinal microbiota in *IL1rn*$^{-/-}$ mice is partly TLR4 dependent. (A-B) Relative abundance of *Ruminococcus*, *Streptococcus*, *Xylanibacter* and Prevotella in wild-type (WT) (n=9), *IL1rn*$^{-/-}$ (n=15), *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ (n=8) and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ (n=8) mice. Data represent relative abundances of these genera obtained by 16S rRNA gene sequencing of the fecal microbiota.

Importantly, production of IL-17 but not IFNγ in lymph nodes draining the inflamed joints was diminished in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice compared with *IL1rn*$^{-/-}$ mice (Supplementary Figure 10). This is in agreement with our previous study showing that TLR4 induces systemic and local IL-17 production and promotes arthritis in *IL1rn*$^{-/-}$ mice [12]. Together these observations suggest an essential role for TLR4 in the induction of intestinal LP IL-17 production associated with extra-intestinal IL-17 levels and the development of arthritis in *IL1rn*$^{-/-}$ mice.

Discussion

The intestinal microbiome has emerged as a key determinant of health and disease. Although advanced sequencing techniques have enabled microbiome profiling in rheumatic patients, study of the underlying mucosal responses and the functional impact on arthritis is limited in human subjects due to the requirement of invasive techniques. The animal studies presented here demonstrate aberrations in intestinal microbiota in mice developing spontaneous autoimmune arthritis, introduce commensal tobramycin-sensitive microbiota as potential triggers for arthritis, and suggest a role for TLR4 activation in mucosal induction of inflammatory pathways including Th17 induction associated with arthritis.

Our study identifies loss of microbial diversity and specific taxonomic alterations in the microbiota of autoimmune-prone *IL1rn*$^{-/-}$ mice. Importantly, loss of intestinal microbial diversity and richness also coincides with human autoimmune diseases such as diabetes, rheumatoid and psoriatic arthritis [14, 16, 43]. Among the microbiota increased in *IL1rn*$^{-/-}$ mice, *Streptococcus* species are known inducers of chronic TLR-mediated arthritis in animal models when injected intra-articularly [44, 45]. Furthermore, a commensal *Helicobacter* (*H. hepaticus*) has been shown to induce IL-23 and mediate T cell-dependent gut inflammation in immunocompromised mice [46]. The decreased *Barnesiella* in *IL1rn*$^{-/-}$ mice is consistent with a previous study associating the abundance of *Barnesiella* with resistance to arthritis in HLA-DRB1*0402 mice [47]. A specific species of *Prevotella*, *P. copri*, is overrepresented in patients with new-onset RA [14], and was recently shown to increase colonic Th17 cells and promote arthritis in SKG mice after co-exposure to the fungal component zymosan [18]. On the other hand, *P. histicola* has been reported to suppress collagen-induced arthritis in transgenic mice expressing RA-susceptibility gene HLA-DQ8 [48]. Therefore, the immunomodulatory effects of the gut microbiota, including *Prevotella*, are species- and sometimes even strain-dependent. Due to inherently limited resolution of 16S rRNA gene sequencing, our data on the abundance of *Prevotella* is limited to the genus level and the exact *Prevotella* species altered in *IL1rn*$^{-/-}$ mice remain unclear. Overall, it is tempting to speculate that complex alterations in several taxa determine the net mucosal response to affect arthritis.

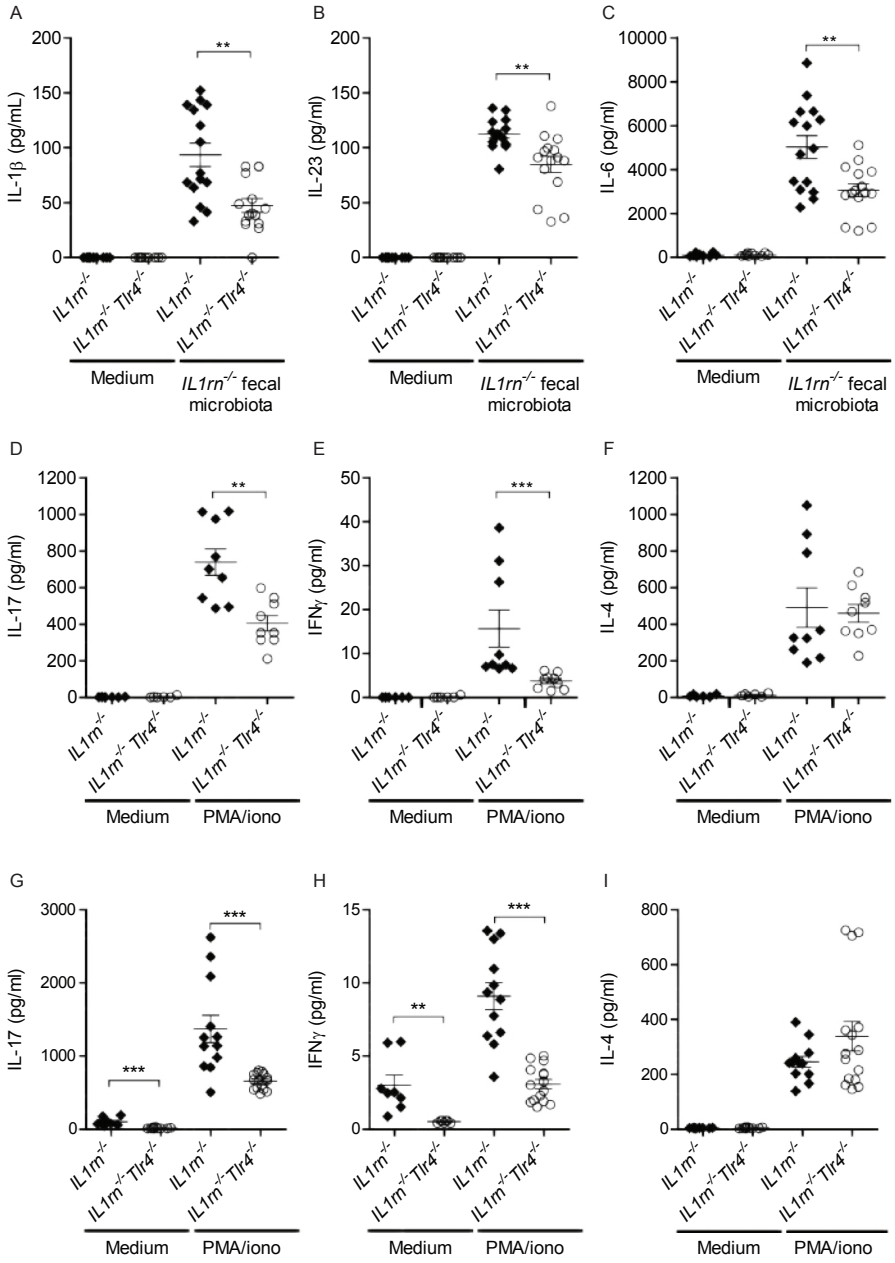


Figure 7. A significant role for TLR4 in intestinal production of cytokines involved in LP Th17 differentiation (A-C) Production of IL-1β, IL-23 and IL-6 by SI-LP mononuclear cells of *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice cultured in the presence of autoclaved *IL1rn*$^{-/-}$ complete fecal microbial antigens for 24 hours.(D-F) Cytokine production by SI-LP lymphocytes of separately-housed *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice *ex vivo* stimulated with PMA and ionomycin for 5 hours. (G-I) Cytokine production by SI-LP lymphocytes of *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice co-housed for 10 days. Cells were stimulated as in D-F. *P ≤0.05, **P ≤ 0.01, ***P ≤ 0.001, by Mann-Whitney U test.

It should also be noted that fecal bacterial community structures do not fully mirror the site-specific luminal or mucosa-associated microbiota profiles and were used in this study as a proxy of the gut microbiota of the *Il1rn*$^{-/-}$ mice.
Our previous studies showed that *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ mice develop a more severe arthritis compared with *IL1rn*$^{-/-}$ mice [12]. Given that additional TLR2 deficiency did not affect the microbiota of *IL1rn*$^{-/-}$ mice to a major extent (Figure 1), we speculate that severe arthritis in *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$ mice is due to the altered host immune response, specifically reduced function of Treg cells [12], rather than alteration in the microbiome. However, the data regarding lack of a major influence of TLR2 deficiency on *Il1rn*$^{-/-}$ microbiota should be interpreted with caution due to the absence of littermate *Il1rn*$^{-/-}$ *Tlr2*$^{+/+}$ mice in our studies.
IL1rn$^{-/-}$ mice had specific expansion of intestinal Th17 cells. The pathogenic relevance of IL-17 in the development of arthritis in *IL1rn*$^{-/-}$ mice has been demonstrated before, since both IL-17 gene deficiency and treatment with neutralizing anti-IL-17 antibodies inhibit arthritis [4, 8]. A previous study showed that γδT cells rather than Th17 cells represent most IL-17-producing T cells in the inflamed joints of *IL1rn*$^{-/-}$ mice [49].While γδ17 and Th17 cells may have complementary pathogenic roles in the development of *IL1rn*$^{-/-}$ arthritis, our data suggest that IL-17-producing cells located in lamina propria and induced by *IL1rn*$^{-/-}$ intestinal microbiota are TCRβ-expressing CD4^{+} Th17 cells (Figure 4 and Supplementary Figure 4).
The expansion of LP Th17 cells in *IL1rn*$^{-/-}$ mice was caused by the dysregulated microbiota as confirmed by fecal transfer experiments (Figure 3G-I). A critical pathogenic link to the spontaneous arthritis was revealed by our germ-free and antibiotic treatment studies (Figure 4 and 5). Other previous studies which demonstrated the involvement of the gut microbiota in exacerbation of autoimmune arthritis found SFB as the responsible microorganisms. One study showed a role for vancomycin-sensitive microbiota including SFB in the induction of IL-17- and autoantibody-driven arthritis in K/BxN mice [13], and another showed that SFB can lower the activation threshold of self-reactive T cells and promote the differentiation of arthritogenic Th1 cells in a T cell transfer model of arthritis [50]. Our data are the first to demonstrate that although SFB colonization exacerbates arthritis, among the dysregulated indigenous microbiota present in the *IL1rn*$^{-/-}$ mice, those sensitive to tobramycin, *i.e., Helicobacter*, *Flexispira*, *Clostridium* and *Dehalobacterium*, are potential candidates to promote arthritis in a genetically susceptible host. This is relevant for human disease, given that SFB were not found in genome-wide sequences of 263 gut metagenomes of human adults [30, 31]. Our data also provide the first evidence for the involvement of TLR4 in defining the intestinal mucosal T cell phenotype. TLR4 activation of LP mononuclear cells by *IL1rn*$^{-/-}$ microbiota induced IL-1β, IL-23 and IL-6 (Figure 7A-C). Microbiota-induced IL-1β is critical for the development of steady-state Th17 cells in the gut [51]. IL-1 also synergizes with IL-6 and IL-23 to regulate early Th17

cells and maintain cytokine expression in effector Th17 cells [52]. It was recently shown that infectious triggers such as influenza lung infection and colitis trigger an IL-1β-induced Th17 differentiation and promote arthritis induced by KRN transgenic T cells [53]. Interestingly, a subset of human $CD14^{+}$ $CD163^{low}$ lamina propria cells expressing both macrophage and DC markers has been found to express TLR4, produce IL-1β and IL-6 upon TLR4 stimulation, and induce Th17 differentiation [54]. However, the specific subset of LP phagocytes that orchestrates the phase-dependent TLR4-mediated mucosal response to microbiota in our studies remains to be determined.

Several studies have shown that TLR4 deficiency and systemic inhibition of TLR4 using specific antagonists or neutralizing antibodies can suppress experimental arthritis [12, 55-57]. Importantly, TLR4 is believed to be hyper-responsive in both blood monocyte-derived DCs and $CD14^{+}$ synovial fluid macrophages of RA patients compared with healthy controls [58, 59]. Pathways associated with TLR signaling are upregulated in synovial fluid macrophages of patients with RA. A proinflammatory role for TLR4 during arthritis has previously been widely attributed to TLR4 activation by endogenous damage-associated molecular patterns present in the joint rather than microbial agonists [12, 57, 60, 61]. Our observations suggest that TLR4-mediated modulation of the mucosal immune response in intestinal LP may be another function involving TLR4 in arthritis.

Conclusions

Our study reveals a crucial role for IL-1Ra in regulation of the diversity and the composition of intestinal microbiota and a balanced T cell response in the intestinal LP. We show that the aberrant microbiota in *IL1rn*$^{-/-}$ mice have the capacity to enhance LP Th17 cells which are associated with arthritis, likely via TLR4-induced production of IL-1β, IL-6 and IL-23. Although *IL1rn*$^{-/-}$ intestinal microbiota do not cause arthritis in a normal (WT) host, these microbiota, in particular tobramycin-sensitive bacteria, contribute to the development of arthritis in *IL1rn*$^{-/-}$ mice. Our data suggest that the interplay between IL-1Ra, intestinal microbiota, TLR4 and mucosal T cells may serve as a potential predisposing or initiating event in the context of autoimmune disease and provide opportunities to control RA.

References

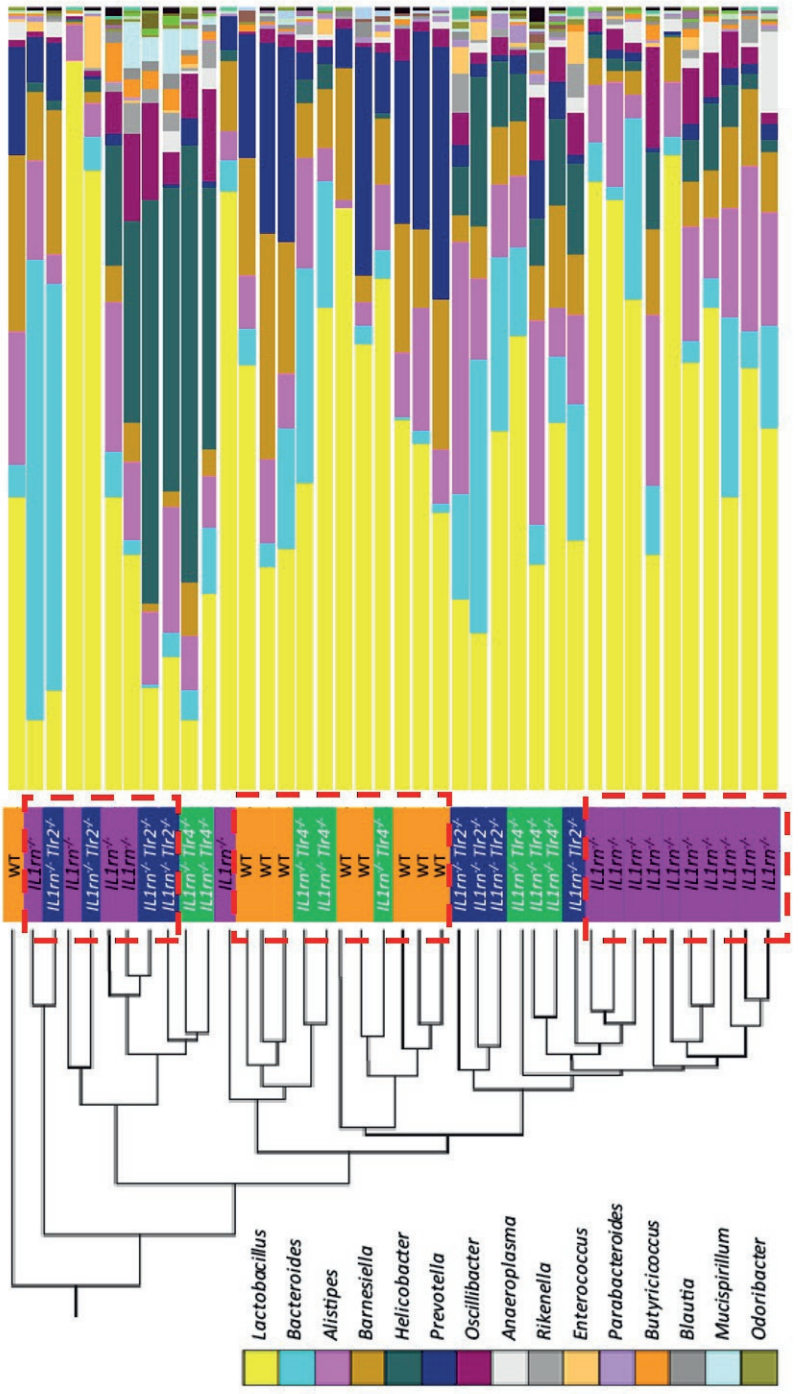
1. Joosten LA, Netea MG, Dinarello CA. Interleukin-1beta in innate inflammation, autophagy and immunity. Seminars in immunology. 2013;25(6):416-24.
2. Carter DB, Deibel MR, Jr., Dunn CJ, Tomich CS, Laborde AL, Slightom JL, et al. Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. Nature. 1990;344(6267):633-8.
3. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. The Journal of experimental medicine. 2000;191(2):313-20.
4. Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(10):5986-90.
5. Shepherd J, Little MC, Nicklin MJ. Psoriasis-like cutaneous inflammation in mice lacking interleukin-1 receptor antagonist. The Journal of investigative dermatology. 2004;122(3):665-9.
6. Matsuki T, Horai R, Sudo K, Iwakura Y. IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions. The Journal of experimental medicine. 2003;198(6):877-88.
7. Matsuki T, Nakae S, Sudo K, Horai R, Iwakura Y. Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis. International immunology. 2006;18(2):399-407.
8. Koenders MI, Devesa I, Marijnissen RJ, Abdollahi-Roodsaz S, Boots AM, Walgreen B, et al. Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice. Arthritis and rheumatism. 2008;58(11):3461-70.
9. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. J Immunol. 2009;183(10):6041-50.
10. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature. 2011;479(7374):538-41.
11. Kriegel MA, Sefik E, Hill JA, Wu HJ, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(28):11548-53.
12. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. The Journal of clinical investigation. 2008;118(1):205-16.
13. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
14. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. eLife. 2013;2:e01202.
15. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nature medicine. 2015;21(8):895-905.
16. Scher JU, Ubeda C, Artacho A, Attur M, Isaac S, Reddy SM, et al. Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease. Arthritis & rheumatology (Hoboken, NJ). 2015;67(1):128-39.
17. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome medicine. 2016;8(1):43.

18. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T cells in the intestine. Arthritis & rheumatology (Hoboken, NJ). 2016.
19. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science (New York, NY). 2012;336(6086):1268-73.
20. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013;500(7461):232-6.
21. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science (New York, NY). 2011;332(6032):974-7.
22. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485-98.
23. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009;31(4):677-89.
24. van den Berg WB, McInnes IB. Th17 cells and IL-17 a--focus on immunopathogenesis and immunotherapeutics. Semin Arthritis Rheum. 2013;43(2):158-70.
25. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. Nature reviews Rheumatology. 2015;11(7):415-29.
26. Genovese MC, Greenwald M, Cho CS, Berman A, Jin L, Cameron GS, et al. A phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. Arthritis & rheumatology (Hoboken, NJ). 2014;66(7):1693-704.
27. Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Aelion JA, et al. One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study. The Journal of rheumatology. 2014;41(3):414-21.
28. Burmester GR, Durez P, Shestakova G, Genovese MC, Schulze-Koops H, Li Y, et al. Association of HLA-DRB1 alleles with clinical responses to the anti-interleukin-17A monoclonal antibody secukinumab in active rheumatoid arthritis. Rheumatology (Oxford, England). 2016;55(1):49-55.
29. Block KE, Zheng Z, Dent AL, Kee BL, Huang H. Gut Microbiota Regulates K/BxN Autoimmune Arthritis through Follicular Helper T but Not Th17 Cells. J Immunol. 2016;196(4):1550-7.
30. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
31. Sczesnak A, Segata N, Qin X, Gevers D, Petrosino JF, Huttenhower C, et al. The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. Cell host & microbe. 2011;10(3):260-72.
32. Nicklin MJ, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. The Journal of experimental medicine. 2000;191(2):303-12.
33. Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PloS one. 2008;3(7):e2836.
34. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 2010;7(5):335-6.
35. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome research. 2011;21(3):494-504.

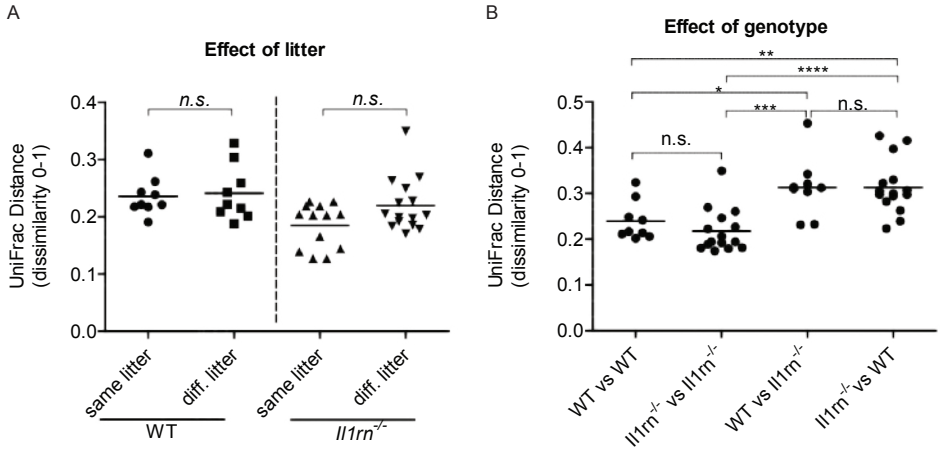
36. QIIME. New default parameters for uclust OTU pickers https://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/2010 [updated December 17, 2010; cited 2016 May 25]. Available from: https://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/.
37. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic acids research. 2009;37(Database issue):D141-5.
38. SciPy.org. SciPy open-source software 2012 [25.05.16]. Available from: http://www.scipy.org/.
39. Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N, et al. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. Infect Immun. 2008;76(3):907-15.
40. Ivanov, II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell host & microbe. 2008;4(4):337-49.
41. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity. 1999;11(4):443-51.
42. Zuniga LA, Jain R, Haines C, Cua DJ. Th17 cell development: from the cradle to the grave. Immunological reviews. 2013;252(1):78-88.
43. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, et al. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal. 2011;5(1):82-91.
44. Joosten LA, Abdollahi-Roodsaz S, Heuvelmans-Jacobs M, Helsen MM, van den Bersselaar LA, Oppers-Walgreen B, et al. T cell dependence of chronic destructive murine arthritis induced by repeated local activation of Toll-like receptor-driven pathways: crucial role of both interleukin-1beta and interleukin-17. Arthritis and rheumatism. 2008;58(1):98-108.
45. Abdollahi-Roodsaz S, Joosten LA, Helsen MM, Walgreen B, van Lent PL, van den Bersselaar LA, et al. Shift from toll-like receptor 2 (TLR-2) toward TLR-4 dependency in the erosive stage of chronic streptococcal cell wall arthritis coincident with TLR-4-mediated interleukin-17 production. Arthritis and rheumatism. 2008;58(12):3753-64.
46. Kullberg MC, Jankovic D, Feng CG, Hue S, Gorelick PL, McKenzie BS, et al. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. The Journal of experimental medicine. 2006;203(11):2485-94.
47. Gomez A, Luckey D, Yeoman CJ, Marietta EV, Berg Miller ME, Murray JA, et al. Loss of sex and age driven differences in the gut microbiome characterize arthritis-susceptible 0401 mice but not arthritis-resistant 0402 mice. PloS one. 2012;7(4):e36095.
48. Luckey, Marietta D, Luthra E, Harvinder S, Patel R, Murray J, et al. Commensal Gut-Derived Bacteria As Therapy for Systemic Autoimmune Disease. Abstracts of the American College of Rheumatology/Association of Rheumatology Health Professionals Annual Scientific Meeting [Internet]. 2012; 64(10). Available from: http://www.blackwellpublishing.com/acrmeeting/abstract.asp?MeetingID=789&id=102367.
49. Akitsu A, Ishigame H, Kakuta S, Chung SH, Ikeda S, Shimizu K, et al. IL-1 receptor antagonist-deficient mice develop autoimmune arthritis due to intrinsic activation of IL-17-producing CCR2(+)Vgamma6(+)gammadelta T cells. Nature communications. 2015;6:7464.
50. Chappert P, Bouladoux N, Naik S, Schwartz RH. Specific gut commensal flora locally alters T cell tuning to endogenous ligands. Immunity. 2013;38(6):1198-210.
51. Shaw MH, Kamada N, Kim YG, Nunez G. Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. The Journal of experimental medicine. 2012;209(2):251-8.
52. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity. 2009;30(4):576-87.

53. Chevalier N, Tan JK, Mason LJ, Robert R, McKenzie CI, Lim F, et al. Avenues to autoimmune arthritis triggered by diverse remote inflammatory challenges. Journal of autoimmunity. 2016;73:120-9.
54. Ogino T, Nishimura J, Barman S, Kayama H, Uematsu S, Okuzaki D, et al. Increased Th17-inducing activity of CD14+ CD163 low myeloid cells in intestinal lamina propria of patients with Crohn's disease. Gastroenterology. 2013;145(6):1380-91.e1.
55. Komai-Koma M, Li D, Wang E, Vaughan D, Xu D. Anti-Toll-like receptor 2 and 4 antibodies suppress inflammatory response in mice. Immunology. 2014;143(3):354-62.
56. van den Brand BT, Abdollahi-Roodsaz S, Bennink MB, Bussink J, Arntz OJ, van den Berg WB, et al. Toll-like receptor 4 in bone marrow-derived cells as well as tissue-resident cells participate in aggravating autoimmune destructive arthritis. Annals of the rheumatic diseases. 2013;72(8):1407-15.
57. Pierer M, Wagner U, Rossol M, Ibrahim S. Toll-like receptor 4 is involved in inflammatory and joint destructive pathways in collagen-induced arthritis in DBA1J mice. PloS one. 2011;6(8):e23539.
58. Huang Q, Ma Y, Adebayo A, Pope RM. Increased macrophage activation mediated through toll-like receptors in rheumatoid arthritis. Arthritis and rheumatism. 2007;56(7):2192-201.
59. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, et al. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. Arthritis and rheumatism. 2005;52(8):2313-22.
60. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, et al. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nature medicine. 2009;15(7):774-80.
61. Abdollahi-Roodsaz S, van de Loo FA, van den Berg WB. Trapped in a vicious loop: Toll-like receptors sustain the spontaneous cytokine production by rheumatoid synovium. Arthritis research & therapy. 2011;13(2):105.

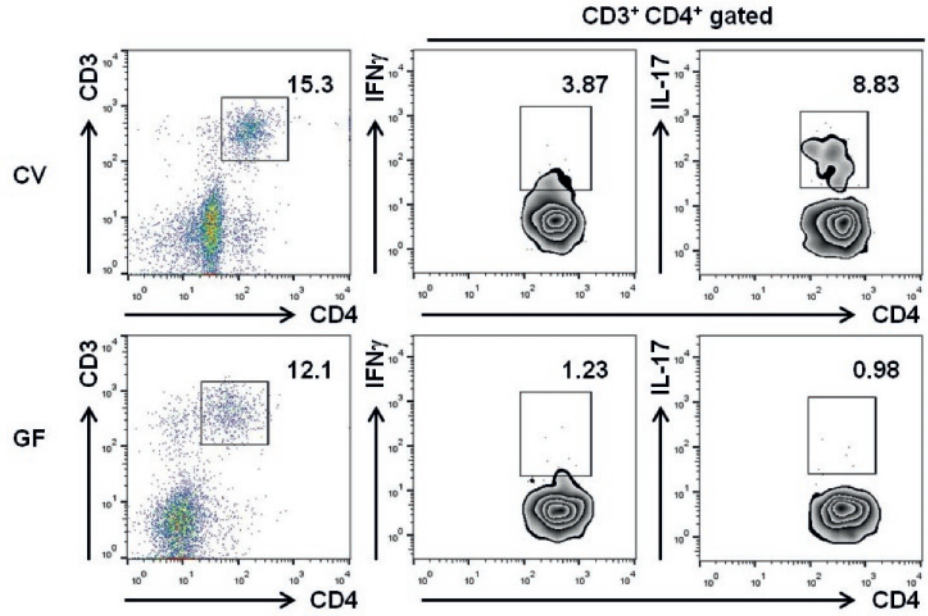
Supplementary material



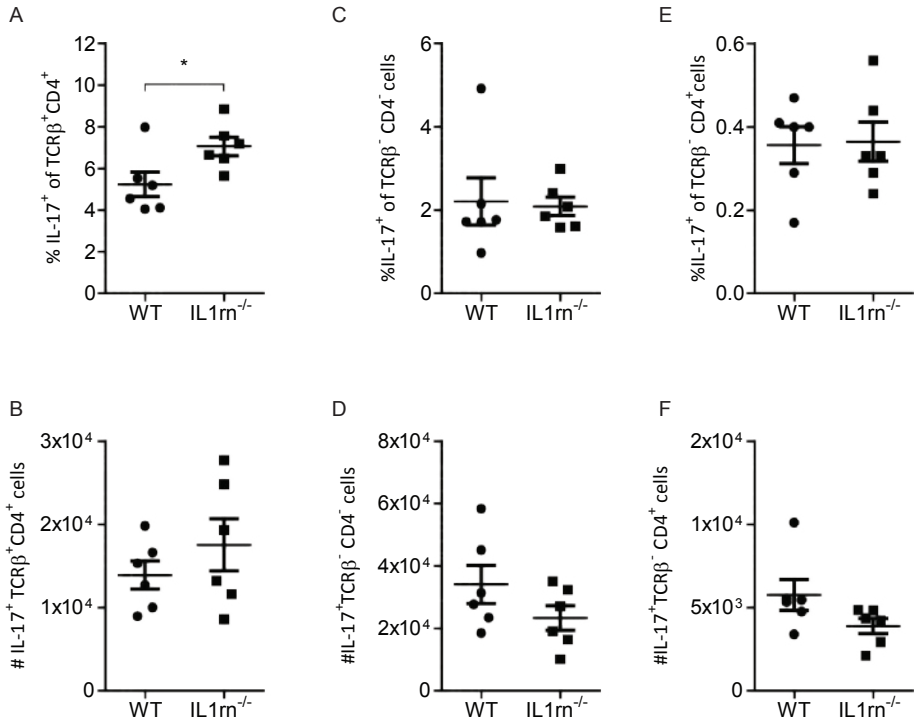
Supplementary Figure 1. Hierarchical clustering of wild-type and *Il1rn*$^{-/-}$ mice based on intestinal microbiota. Hierarchical weighed UniFrac clustering of fecal samples of WT (n=9), *Il1rn*$^{-/-}$ (n=15), *Il1rn*$^{-/-}$ *Tlr2*$^{-/-}$ (n=8) and *Il1rn*$^{-/-}$ *Tlr4*$^{-/-}$ (n=8) mice based on their intestinal microbial composition. Data were obtained from 16S rRNA gene 454-pyrosequencing of fecal bacterial DNA of 15-week-old mice. The dashed red lines highlight the mice clustered together based on their intestinal microbiota.



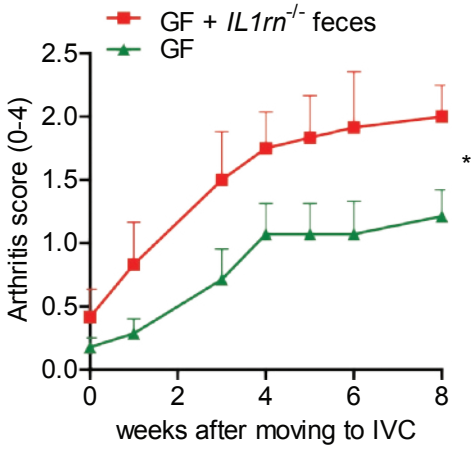
Supplementary Figure 2. The impact of lineage origin (A) versus *IL1rn*-deficiency (B) on the overall fecal microbiota composition. (A) To assess the effects of lineage origin and caging, the weighted UniFrac distance was calculated for each mouse in a colony versus all other mice of the same genotype in the same litter (intra-cage distances) and all other mice of the same genotype from a different litter (inter-cage effects). (B) The effect of genotype (WT or *IL1rn*$^{-/-}$) is shown as the weighted UniFrac distance for each mouse versus all other mice from either the same or the opposite genotype. A higher UniFrac distance indicates greater dissimilarity between the microbial communities. Error bars indicate mean ± SEM. n.s. not significant; *P ≤ 0.05, **P ≤ 0.01, and *** P ≤ 0.001 by Mann-Whitney U test.



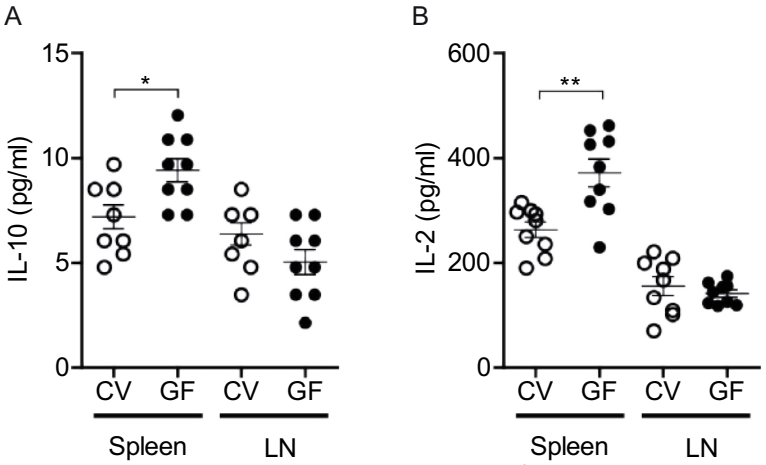
Supplementary Figure 3. Gating strategy. Flow cytometry gating strategy used to identify IFNγ and IL-17 producing CD3^{+}CD4^{+} T cells in small intestine lamina propria of conventional (CV) and germ-free (GF) *IL1rn*$^{-/-}$ mice.



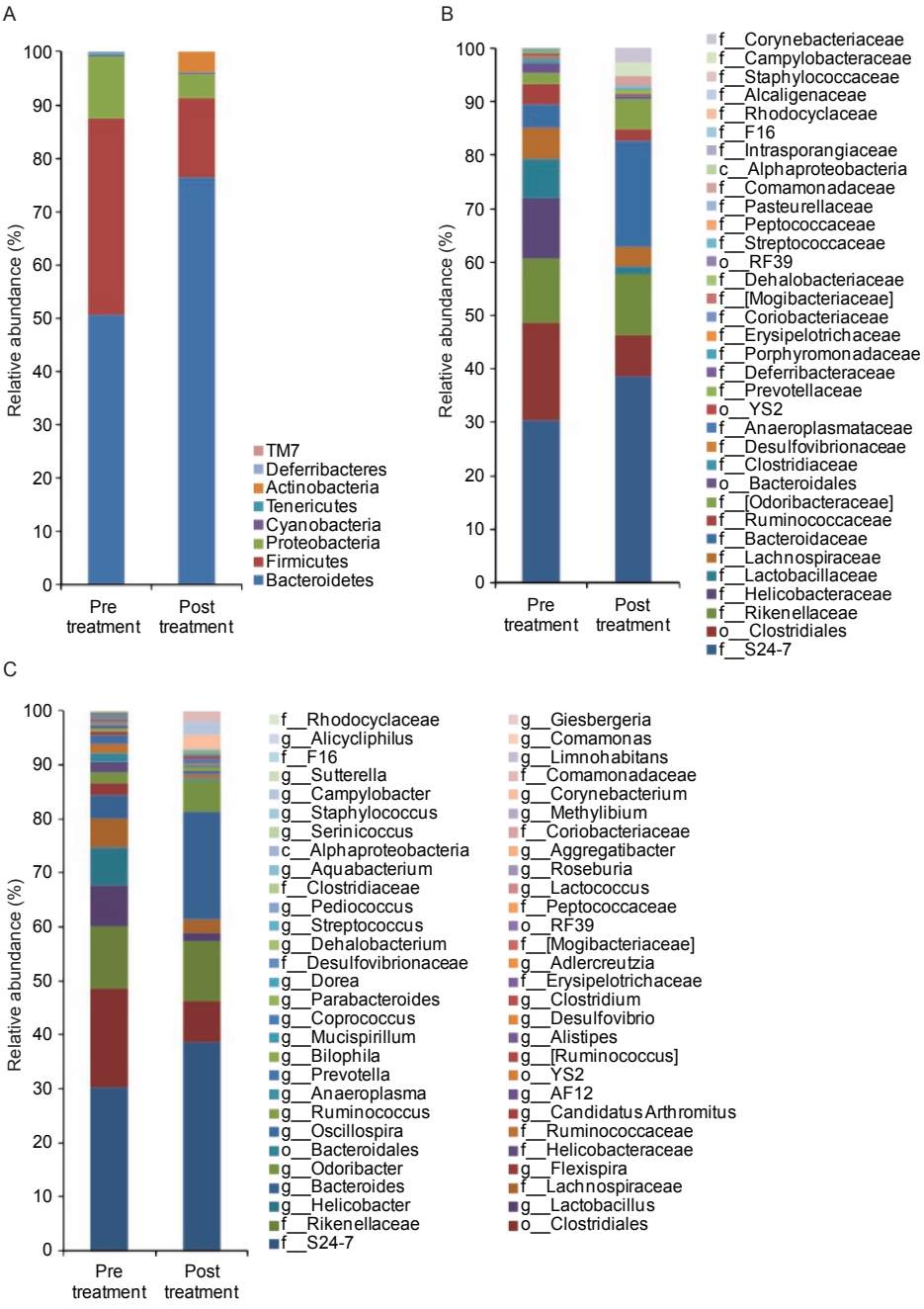
Supplementary Figure 4. Frequencies and numbers of IL-17-producing cells among TCRβ⁺ and TCRβ⁻ T cell populations with and without CD4 expression. Only the proportion of TCRβ⁺ CD4⁺ IL-17⁺ (Th17) cells shows significant increase in lamina propria of *IL1rn*$^{-/-}$ mice. Error bars indicate mean ± SEM. *P ≤ 0.05 by Mann-Whitney U test.



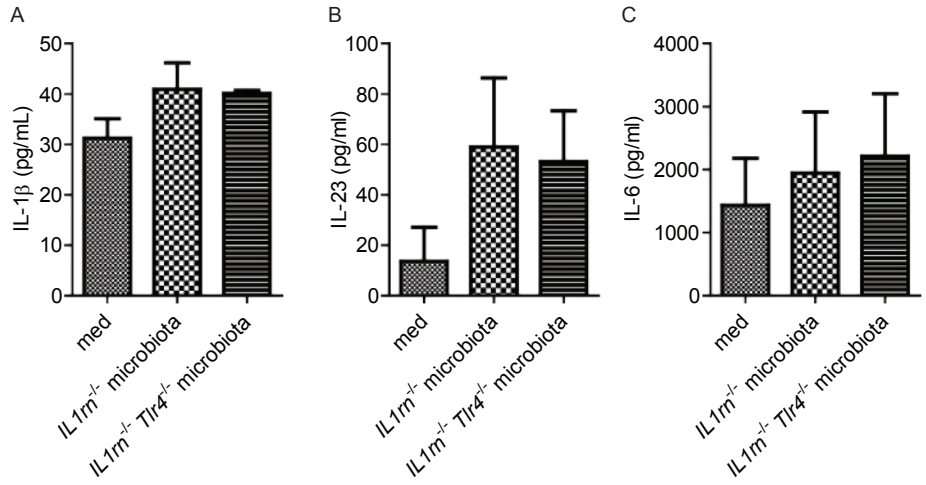
Supplementary Figure 5. Colonization of germ-free (GF) *IL1rn*$^{-/-}$ mice with fecal microbiota of conventional *IL1rn*$^{-/-}$ mice increases the severity of arthritis. GF *IL1rn*$^{-/-}$ mice received either 200 µl of sterile water or 200 µl fecal suspension of conventional *IL1rn*$^{-/-}$ mice and were monitored for the development of arthritis for 8 weeks. *P ≤ 0.05.



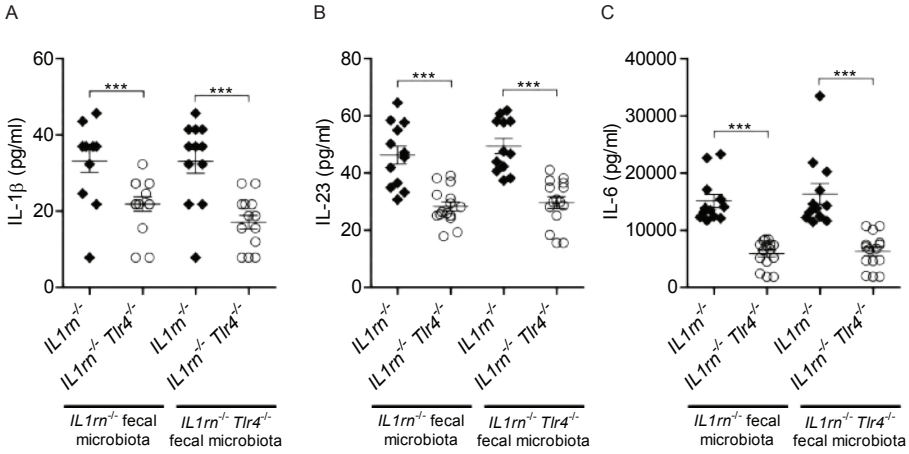
Supplementary Figure 6. Increased expression of Th2/Treg cytokines in spleens but not popliteal lymph nodes (LN) of germ-free *IL1rn*$^{-/-}$ mice. (A-B) Production of IL-10 and IL-2 upon *ex vivo* stimulation of spleen and lymph node cells from conventional (CV) and germ-free (GF) mice with PMA and ionomycin for 6 hours, as measured by Luminex assay. n=3 mice per group each stimulated in triplicate. n.s. not significant, *P ≤ 0.05 and **P ≤ 0.01, by Mann-Whitney U test.



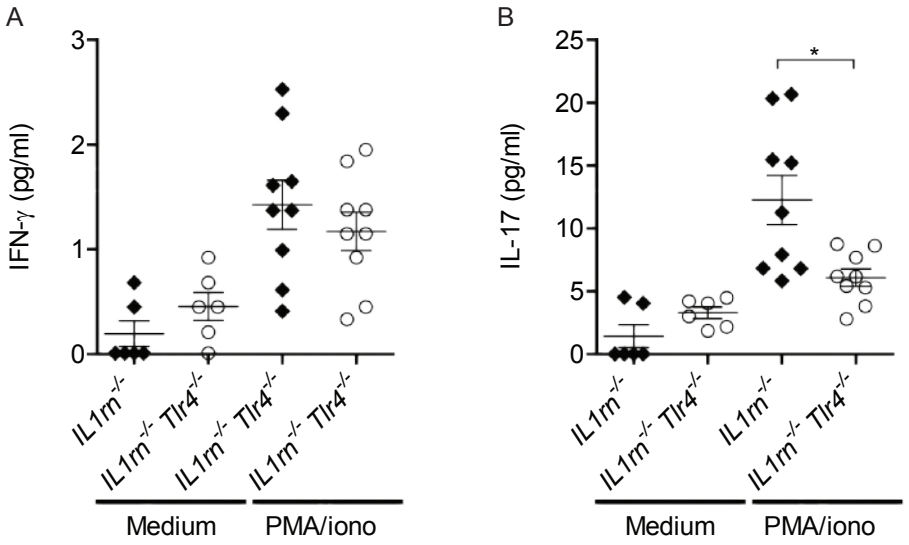
Supplementary Figure 7. Effects of 8 weeks oral tobramycin treatment on microbiota of *IL1rn*$^{-/-}$ mice assessed by 16S gene sequencing of fecal bacterial DNA**.** Fecal pellets were collected at the baseline and at the end-point (8 weeks) of treatment. o=order, f= family and g=genus.



Supplementary Figure 8. *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ microbiota induce similar cytokine response in lamina propria mononuclear cells. Production of IL-1β, IL-23 and IL-6 by small intestine lamina propria mononuclear cells of *IL1rn*$^{-/-}$ mice cultured in the presence of autoclaved *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ complete fecal microbial antigens (1:200 v/v ratio) for 24 hours. n.s., not significant.



Supplementary Figure 9. Lamina propria mononuclear cells of *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice co-housed with *IL1rn*$^{-/-}$ mice produce less Th17-inducing cytokines. Production of IL-1β, IL-23 and IL-6 by lamina propria mononuclear cells of *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice co-housed for 10 days. Cells were stimulated for 24 hours with fecal microbial antigens from (separately housed) *IL1rn*$^{-/-}$ and *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice. ***P ≤ 0.001 by Mann-Whitney U test.



Supplementary Figure 10. Decreased IL-17 production in draining lymph nodes of TLR4 deficient mice. (A-B) Cytokine production by draining lymph node cells of *IL1rn*$^{-/-}$ *and IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice *ex vivo* stimulated with PMA and ionomycin for 5 hours.

Supplementary Table 1. The average and total number of (assigned) reads and operational taxonomic units (OTU) per experimental group. In addition, the number and percentage of reads assigned to phylum or genus level are shown.

	Reads			OTU			Assigned at Phylum		Assigned at Genus		
Genotype	Average	SEM	Total	Average	SEM	Total	Total reads	%	Total reads	%	Group size
WildType	4286	626	38576	617	57	5554	37890	98.2	15159	39.3	n=9
IL1rn$^{-/-}$	5947	462	89200	524	28	7854	88539	99.3	37284	41.8	n=15
IL1rn$^{-/-}$*Tlr2*$^{-/-}$	5897	432	47179	661	41	5291	46461	98.5	17338	36.7	n=8
IL1rn$^{-/-}$*Tlr4*$^{-/-}$	9364	936	74910	1120	74	8957	73565	98.2	26601	31.5	n=8

Supplementary Table 2. TLR4 deficiency normalizes specific aberrations in *Il1rn*$^{-/-}$ intestinal microbiome towards WT level. A full list of significantly altered microbial taxa in *IL1rn*$^{-/-}$ mice compared to WT controls and the taxa normalized in *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$ mice. Significant alterations by Mann-Whitney U test are highlighted in light green and those significant after Bonferroni correction are highlighted in dark green.

	Relative abundance			Fold Change	
	WT	IL1rn$^{-/-}$	IL1rn$^{-/-}$ Tlr4$^{-/-}$	WT vs. *IL1rn*$^{-/-}$	IL1rn$^{-/-}$ vs. *IL1rn*$^{-/-}$ *Tlr4*$^{-/-}$
phylum Actinobacteria	**0.31%**	**0.15%**	**0.25%**	**-1.01**	**0.65**
phylum Bacteroides	41.84%	23.10%	26.04%	-0.86	n.s.
phylum Deferribacteres	0.03%	0.03%	0.09%	0.84	1.5
phylum Firmicutes	55.52%	73.70%	68.14%	0.42	n.s.
phylum Proteobacteria	0.34%	1.49%	3.35%	1.95	n.s.
phylum TM7	0.03%	0.04%	0.07%	n.s.	0.72
class Actinobacteria	**0.31%**	**0.15%**	**0.25%**	**-1.01**	**0.65**
class Bacilli	19.62%	27.98%	17.29%	n.s.	n.s.
class Bacteroidia	39.77%	22.19%	25.30%	-0.85	n.s.
class Betaproteobacteria	0.09%	0.00%	0.00%	-10	n.s.
class Clostridia	34.62%	44.07%	48.60%	n.s.	n.s.
class Deferribacteres	0.03%	0.03%	0.09%	0.84	1.5
class Deltaproteobacteria	0.09%	0.10%	0.27%	n.s.	n.s.
class Epsilonproteobacteria	0.00%	1.37%	3.03%	10	n.s.
order Bacillales	0.00%	0.02%	0.04%	10	n.s.
order Bacteroidales	39.77%	22.19%	25.30%	-0.85	n.s.
order Burkholderiales	0.09%	0.00%	0.00%	-10	n.s.
order Campylobacterales	0.00%	1.37%	3.03%	10	n.s.
order Clostridiales	34.15%	43.93%	48.38%	n.s.	n.s.
order Coriobacteriales	**0.29%**	**0.14%**	**0.24%**	**-1**	**0.7**
order Deferribacterales	0.03%	0.03%	0.09%	0.84	1.5
order Desulfovirbrionales	0.09%	0.10%	0.27%	n.s.	n.s.
order Lactobacillales	18.85%	27.33%	16.67%	n.s.	n.s.
family Alcaligenaceae	0.09%	0.00%	0.00%	-10	n.s.
family Bacteroidaceae	1.05%	3.66%	4.00%	n.s.	n.s.
family Coriobacteriaceae	**0.29%**	**0.14%**	**0.24%**	**-1**	**0.7**
family Deferribacteraceae	0.03%	0.03%	0.09%	0.84	1.5
family Desulfovibrionaceae	0.08%	0.09%	0.17%	n.s.	n.s.
family Enterococcaceae	0.03%	0.05%	0.19%	n.s.	n.s.
family Helicobacteraceae	0.00%	1.37%	3.00%	10	n.s.
family Incertae Sedis XIV	0.16%	0.09%	0.11%	n.s.	n.s.
family Lactobacillaceae	17.52%	26.30%	15.37%	n.s.	n.s.
family Peptococcaceae	0.01%	0.03%	0.01%	1.43	n.s.
family Porphyromonadaceae	13.82%	5.64%	5.17%	-1.27	n.s.
family Prevotellaceae	**10.85%**	**0.68%**	**3.05%**	**-3.97**	**2.17**
family Rikenellaceae	4.27%	5.05%	3.48%	n.s.	n.s.
family Ruminococcaceae	2.42%	2.68%	3.55%	n.s.	0.45
family Staphylococcaceae	0.00%	0.02%	0.04%	10	n.s.
family Streptococcaceae	**0.02%**	**0.11%**	**0.04%**	**2.58**	**-1.23**

	Relative abundance			Fold Change	
	WT	IL1rn$^{-/-}$	IL1rn$^{-/-}$ Tlr4$^{-/-}$	WT vs. IL1rn$^{-/-}$	IL1rn$^{-/-}$ vs. IL1rn$^{-/-}$ Tlr4$^{-/-}$
genus *Acetivibrio*	0.01	0.01	0.02	n.s.	n.s.
genus *Alistipes*	3.92	4.48	3.03	n.s.	n.s.
genus *Anaerofilum*	0.00	0.00	0.02	n.s.	10
genus *Bacteroides*	1.05	3.66	4.00	n.s.	n.s.
genus *Barnesiella*	6.44	2.53	2.34	-1.35	n.s.
genus *Blautia*	0.16	0.09	0.11	n.s.	n.s.
genus *Butyricicoccus*	0.12	0.19	0.12	n.s.	n.s.
genus *Butyricimonas*	0.00	0.02	0.01	10	n.s.
genus *Enterococcus*	0.03	0.05	0.18	n.s.	n.s.
genus *Enterorhabdus*	0.03	0.02	0.04	n.s.	1.27
genus *Helicobacter*	0.00	1.30	2.85	10	n.s.
genus *Lactobacillus*	17.51	26.29	15.35	n.s.	n.s.
genus *Mucispirillum*	0.03	0.03	0.09	0.84	1.5
genus *Parabacteroides*	0.13	0.08	0.26	n.s.	1.54
genus *Parasutterella*	0.09	0.00	0.00	-10	n.s.
genus *Peptococcus*	0.01	0.03	0.01	1.43	n.s.
genus *Prevotella*	**8.42**	**0.60**	**1.19**	**-3.71**	**0.95**
genus *Rikenella*	0.03	0.29	0.20	3.7	n.s.
genus *Ruminococcus*	**0.11**	**0.02**	**0.09**	**-2.72**	**2.34**
genus *Sporobacter*	0.03	0.00	0.01	n.s.	3.71
genus *Staphylococcus*	0.00	0.02	0.04	10	n.s.
genus *Streptococcus*	**0.02**	**0.10**	**0.03**	**2.44**	**-1.57**
genus *TM7*	0.03	0.04	0.07	n.s.	0.72
genus *Wolinella*	0.00	0.01	0.04	10	n.s.
genus *Xylanibacter*	**0.09**	**0.00**	**0.07**	**-6.95**	**6.34**
species *Bacteroides acidofaciens*	0.86	2.10	0.92	n.s.	n.s.
species *Bacteroides vulgatus*	0.10	0.00	0.00	-5.85	n.s.
species *Barnesiella intestinihominis*	0.06	0.01	0.03	n.s.	1.52
species *Clostridium sp*	0.00	0.00	0.02	n.s.	10
species *Enterococcus faecium*	0.03	0.05	0.18	n.s.	n.s.
species *Helicobacter hepaticus*	0.00	0.00	0.02	n.s.	10
species *Lactobacillus acidophilus*	0.00	0.39	0.37	10	n.s.
species *Lactobacillus johnsonii*	5.97	11.72	5.28	n.s.	n.s.
species *Lactobacillus reuteri*	7.01	10.45	4.57	n.s.	-1.17
species *mpn isolate group*	0.10	0.08	0.02	n.s.	-2.13
species *Parabacteroides distasonis*	0.04	0.00	0.01	-10	n.s.
species *Parabacteroides merdae*	0.00	0.05	0.06	10	n.s.
species *Ruminococcus flavefaciens*	**0.11**	**0.02**	**0.09**	**-2.72**	**2.32**
species *Ruminococcus gnavus*	0.02	0.00	0.06	n.s.	5.01
species *Staphylococcus cohnii*	0.00	0.02	0.04	10	n.s.

Supplementary Table 3. Assessment of the presence of SFB expression in WT, *IL1rn*$^{-/-}$, *IL1rn*$^{-/-}$ *Tlr2*$^{-/-}$, *IL1rn*$^{-/-}$*Tlr4*$^{-/-}$ mice. The Ct (cycle threshold) value for SFB-specific 16S rRNA gene by qPCR is shown. The delta Ct (ΔCt) value was calculated for SFB-specific 16S rRNA gene relative to the total (conserved) bacterial 16S rRNA genes amplified using universal bacterial primers. Data are presented as relative SFB expression calculated as $2^{-\Delta Ct}$ x 10,000. Mean ± SEM per experimental group is shown.

Genotype	Number of mice	Number of mice with detectable SFB DNA by qPCR	Range of Ct value for SFB DNA by SFB-specific qPCR	ΔCt (Ct by SFB primers - Ct by universal primers	Relative SFB DNA DNA corrected for universal bacterial 16S DNA ($2^{-\Delta Ct}$ x 10,000)
WildType	9	9	31.2 - 36.3	15.49 ± 0.64	0.52 ± 0.23
IL1rn$^{-/-}$	15	10	32.6 - undetectable	18.29 ± 0.85	0.22 ± 0.14
IL1rn$^{-/-}$*Tlr2*$^{-/-}$	8	6	28.4 - undetectable	17.60 ± 2.01	0.31 ± 0.25
IL1rn$^{-/-}$*Tlr4*$^{-/-}$	8	6	32.8 - undetectable	19.79 ± 1.44	0.078 ± 0.047

Supplementary Table 4. Alterations in fecal microbiota with a relative abundance > 0.1% by oral tobramycin, sorted by the abundance at the baseline. Fecal DNA samples at baseline and at 8 weeks post-tobramycin treatment are compared. Significant alterations by Mann-Whitney U test are highlighted in green and those significant after Bonferroni correction for multiple testing are highlighted in blue. N=9 mice per group.

	Baseline		Post-tobramycin treatment	
	Mean	**SEM**	**Mean**	**SEM**
family S24-7	30.367	5.990	38.709	6.606
order Clostridiales	18.373	4.275	7.707	1.731
family Rikenellaceae	11.375	1.275	11.001	2.964
genus *Lactobacillus*	7.596	1.892	1.402	0.572
genus *Helicobacter*	7.181	2.991	0.001	0.001
family Lachnospiraceae	5.313	0.997	2.595	1.042
genus *Bacteroides*	4.254	1.178	20.061	3.936
genus *Flexispira*	2.246	0.496	0.000	0.000
genus *Odoribacter*	2.105	0.375	5.750	2.088
family Helicobacteraceae	1.819	0.710	0.000	0.000
order Bacteroidales	1.717	0.549	0.458	0.141
family Ruminococcaceae	1.698	0.515	0.672	0.269
genus *Oscillospira*	1.645	0.457	0.611	0.147
genus *Ruminococcus*	0.539	0.153	0.757	0.228
genus *AF12*	0.419	0.065	0.297	0.121
genus *Anaeroplasma*	0.357	0.141	0.180	0.078
order YS2	0.346	0.125	0.096	0.061
genus *Prevotella*	0.340	0.109	0.778	0.168
genus [*Ruminococcus*]	0.258	0.093	0.448	0.137
genus *Bilophila*	0.232	0.097	0.010	0.008
genus *Alistipes*	0.161	0.036	0.083	0.043
genus *Mucispirillum*	0.155	0.098	0.000	0.000
genus *Desulfovibrio*	0.120	0.053	0.007	0.005
genus *Clostridium*	0.107	0.036	0.000	0.000
genus *Coprococcus*	0.104	0.041	0.425	0.127
genus *Parabacteroides*	0.092	0.038	0.277	0.086
family Erysipelotrichaceae	0.072	0.029	0.038	0.020
genus *Dorea*	0.051	0.021	0.145	0.062
genus *Dehalobacterium*	0.049	0.015	0.000	0.000
family [Mogibacteriaceae]	0.043	0.013	0.000	0.000
family Desulfovibrionaceae	0.042	0.013	0.030	0.025
genus *Adlercreutzia*	0.042	0.019	0.000	0.000
order RF39	0.023	0.023	0.000	0.000
family Peptococcaceae	0.017	0.005	0.000	0.000
genus *Streptococcus*	0.007	0.004	0.293	0.293
genus *Aquabacterium*	0.003	0.003	0.022	0.022
genus *Corynebacterium*	0.000	0.000	2.642	2.642
genus *Campylobacter*	0.000	0.000	2.452	2.452
family Comamonadaceae	0.000	0.000	1.952	1.952
genus *Sutterella*	0.000	0.000	0.024	0.018
genus *Limnohabitans*	0.000	0.000	0.018	0.018

Chapter 4

Alteration of the intestinal microbiome characterizes preclinical inflammatory arthritis in mice and its modulation attenuates established arthritis

Chapter 4

Alteration of the intestinal microbiome characterizes preclinical inflammatory arthritis in mice and its modulation attenuates established arthritis

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Abstract

Perturbations of the intestinal microbiome have been observed in patients with new-onset and chronic autoimmune inflammatory arthritis. However, it is currently unknown whether these alterations precede the development of arthritis or are rather a consequence of disease. Modulation of intestinal microbiota by oral antibiotics or germ-free condition can prevent arthritis in mice. Yet, the therapeutic potential of modulation of the microbiota after the onset of arthritis is not well characterized. We here show that the intestinal microbial community undergoes marked changes in the preclinical phase of collagen induced arthritis (CIA). The abundance of the phylum Bacteroidetes, specifically families S24-7 and Bacteroidaceae was reduced, whereas Firmicutes and Proteobacteria, such as Ruminococcaceae, Lachnospiraceae and Desulfovibrinocaceae, were expanded during the immune-priming phase of arthritis. In addition, we found that the abundance of lamina propria Th17, but not Th1, cells is highly correlated with the severity of arthritis. Elimination of the intestinal microbiota during established arthritis specifically reduced intestinal Th17 cells and attenuated arthritis. These effects were associated with reduced serum amyloid A expression in ileum and synovial tissue. Our observations suggest that intestinal microbiota perturbations precede arthritis, and that modulation of the intestinal microbiota after the onset of arthritis may offer therapeutic opportunities.

Introduction

Commensal intestinal microbiota have been implicated in several autoimmune diseases including psoriasis, psoriatic arthritis (PsA) and rheumatoid arthritis (RA) [1, 2]. PsA and RA are systemic autoimmune diseases characterized by chronic joint inflammation and progressive damage to bone and cartilage. Although their exact etiologies are unknown, both diseases are considered multifactorial and driven by a combination of genetic and environmental factors [3, 4]. Several recent studies have shown that the composition of intestinal microbiota is perturbed in patients with recent-onset PsA [5] and new-onset as well as chronic RA [6-9]. PsA microbiota is characterized by a significant reduction in *Akkermansia, Ruminococcus, and Pseudobutyrivibrio* [5]. Two studies in patients with new-onset RA have reported significant expansion of *Prevotella copri* prior to immunosuppressive treatment [6, 8]. Another study found enrichment of *Lactobacillus salivarius* in RA fecal microbiota, especially during very active disease [7]. This study also found a cluster of metagenomic linkage groups related to *Clostridium asparagiforme*, *Gordonibacter pamelaeae*, *Eggerthella lenta* and Lachnospiraceae to be associated with RA with varying duration (3 months to >10 years) [7]. A fourth study in patients with longstanding, treated RA (mean disease duration 81.6 months) demonstrated increased abundance of *Collinsella*, *Eggerthella*, and *Faecalibacterium* [9]. Despite this increasing evidence of the altered microbiota in patients with RA and PsA, it is not clear whether the observed perturbations in the intestinal microbiome precede the development of clinical arthritis. Furthermore, the involvement of the microbiome and its targetability during ongoing inflammatory arthritis is less well understood.

One of the most prominent effects of microbiota is to define the balance between the pro-inflammatory $CD4^+$ T helper 1 (Th1) and Th17 cells and protective regulatory T (Treg) cells, both at mucosal surfaces and systemically [10, 11]. Th17 cells are considered to play a pathogenic role in PsA and RA by producing several proinflammatory cytokines such as interleukin-17 (IL-17), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor-necrosis factor (TNFα) [12-16]. Blockade of IL-17A and its receptor is highly efficacious in the treatment of PsA [17, 18] and, despite lack of robust efficacy in RA, can induce sustained American College of Rheumatology 50 (ACR50) response in a subset of patients with RA [19-21]. Th17 cells also induce the production of IL-6, IL-8 and tissue-destructive matrix metalloproteinases by other cells such as macrophages and fibroblasts [13, 22-25]. Therefore, modulation of the intestinal microbiome may suppress inflammatory arthritis by reducing Th17 cells and multiple Th17-related related proinflammatory mediators.

Several studies have investigated the role of intestinal microbiota in the onset of inflammatory arthritis in mouse models. Independent lines of evidence have

demonstrated that the development of spontaneous arthritis in SKG, K/BxN and IL-1 receptor antagonist deficient (IL-1Ra$^{-/-}$) mice is abrogated under germ-free (GF) conditions [26-28]. Colonization of GF K/BxN mice with segmented filamentous bacteria (SFB) reinstated Th17 cell differentiation and the production of disease-inducing autoantibodies, and accelerated the onset of arthritis [28]. It was recently shown that SFB stimulate Th17 differentiation via induction of serum amyloid A (SAA) 1 and SAA2 production by intestinal epithelial cell [29, 30]. IL-22 secretion by type 3 innate lymphoid cells (ILC3) was shown to be essential for SAA production by intestinal epithelial cells [30]. In addition, it was shown that inoculating GF arthritis-prone SKG mice with *Prevotella*-dominated microbiota of RA patients resulted in increased numbers of intestinal Th17 cells and enhanced the development of arthritis compared with mice receiving fecal microbiota from healthy controls [8].

Importantly, depletion of intestinal microbiota with broad-spectrum antibiotics before the induction of antigen-induced arthritis and experimental autoimmune encephalomyelitis reduced disease severity [31, 32]. A recent study showed that colonizing mice with the human gut commensal *Prevotella histicola* suppressed Th17 responses and the development of inflammatory arthritis in mice [33]. These observations suggest that microbiota-induced modulation of Th17 cell differentiation affects the initial development of inflammatory arthritis in mice. However, the role of the intestinal microbiota in the progression of established arthritis and the involved processes are unknown.

Using high-throughput sequencing of bacterial 16S rRNA, we here show that marked changes in the intestinal microbiota occur in the preclinical phase of inflammatory arthritis and precede the onset of the disease in mice. In addition, we show that modulation of intestinal microbiota during ongoing CIA alters both intestinal and joint-adjacent T cell profiles associated with changes in SAA and IL-22 expression, and can serve as a potential means to control the progression of established inflammatory arthritis.

Material and methods

Mice and induction of CIA

Male DBA/1J mice (Janvier, France) were housed in individually-ventilated cages, and water and food were provided *ad libitum*. CIA was induced by intradermal injection of 100 µg bovine type II collagen (CII) in Freund's complete adjuvant on day 0 and intraperitoneal booster injection of 100 µg CII in PBS on day 21. Clinical onset and progression of arthritis was scored on a scale between 0-2 for each paw as described before [34]. All animal studies were approved by the institutional review board (Animal Experimentation Committee of Radboud University Medical Center) and were conducted in accordance with the institutional guidelines.

Sample collection and DNA extraction
Feces were collected and stored at -80°C until processing. Fecal DNA was collected using the PowerLyzer DNA isolation kit (MO BIO laboratories) following manufacturer's instructions.

16S sequencing of intestinal microbiota
Amplicon library preparation was performed using an automated platform (Biomek 4000) with a custom liquid handling method. For each sample, the V4 region of the bacterial 16S rRNA gene was amplified in duplicate reactions using primer set 515F/806R, which nearly universally amplifies bacterial and archaeal 16S rRNA genes. Each unique barcoded amplicon was generated in pairs of 25 µl reactions with the following reaction conditions: 11 µl PCR-grade H2O, 10 µl Hot MasterMix, 2 µl of forward and reverse barcoded primer (5 µM) and 2 µl template DNA. Reactions were run on a C1000 Touch Thermal Cycler (Bio-Rad) with the following cycling conditions: initial denaturing at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 1 minute, and extension at 72°C for 90 seconds, with a final extension of 10 minutes at 72°C. Amplicons were quantified using the Agilent 2200 TapeStation system and pooled. Purification was then performed using Ampure XT (Beckman Coulter) as per the manufacturer instructions. Sequencing was performed using the MiSeq (Illumina) platform to produce 150 base-pair end reads and define the microbiota composition, as we previously described [35]. The obtained 16S rRNA sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline for analysis of community sequence data. Briefly, reads were demultiplexed and quality filtered with default parameters using prinseq. Sequences were then clustered into operational taxonomic units (OTUs) using a 97% similarity threshold with USEARCH and the Greengenes 16S reference dataset and taxonomy [36]. PyNAST was used as the default alignment tool for QIIME.

Antibiotic treatment
CIA mice with a macroscopic score of 0.25-2.75 were randomized for treatment with a cocktail of 0.5 g/L vancomycin (Sigma), and 1 g/L metronidazole (Acros Organics), neomycin trisulfate (Sigma) and ampicillin sodium salt (Sigma) provided in drinking water containing 6 g/L sucrose for 1 week or for the control treatment (only sucrose).

Isolation of lamina propria-lymphocytes (LPLs)
Small intestine and colon were isolated and residual mesenteric fat and Peyer's patches were removed. The intestine was opened longitudinally, washed with ice-cold PBS and treated twice with 5 mM EDTA for 10 minutes at 37°C to remove the epithelial cells. The tissue was further digested using collagenase D

(0.5 mg/ml, Roche), DNase I (0.25 mg/ml, Sigma), and Dispase (50 U/ml, Fisher) for 3 cycles of 20 minutes at 37°C. Cells were passed through a 40 µm cell strainer and LP lymphocytes were harvested at the interphase of a 40%/80% Percoll gradient (Sigma).

Induction of K/BxN serum-transfer arthritis

Serum-transfer arthritis was induced in C57Bl/6 mice by two intraperitoneal injections of 200µl arthritogenic serum derived from arthritic K/BxN mice on days 0 and 2, as described before [37]. Mice were treated with antibiotics as described above for CIA starting from day 0 because of the acute nature and short span of the serum-transfer model.

Flow cytometry

Cells were incubated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma), ionomycin (1 µg/ml; Sigma), and Brefeldin A (1 µl/ml; BD Biosciences) at 37°C for 4 hours and stained with anti-CD4-APC (Biolegend), anti-TCRβ-FITC (Biolegend) and fixable viability dye (eBioscience). For intracellular staining, cells were fixed and permeabilized using fixation/permeabilization buffer (eBioscience) and stained with anti-IL-17-PECy7 (biolegend), anti-IFNγ-PE (BD pharmingen), and anti-FOXP3-PE (eBioscience) in permeabilization buffer (eBioscience). Data were collected on Gallios flow cytometer (Beckman Coulter) and analyzed with Flowjo 10.0 software.

Cell culture and measurement of cytokines

Popliteal lymph nodes (pLN) were disrupted on a 70 µm cell strainer. LPLs (4 x 10^5 cells/well) and pLN cells (2 x 10^5 cells/well) were cultured with PMA (50 ng/ml; sigma) and ionomycin (1µg/ml; Sigma) for 6 hours. Cytokine concentrations in culture supernatants and mice sera were measured by Luminex using the mouse cytokine/chemokine magnetic bead kits (Bio-Rad).

RNA isolation and quantitative real-time PCR

Tissues were homogenized using a MagNA lyser instrument (Roche). RNA was isolated in TRIzol reagent (Sigma) as described before [27]. Quantitative real-time PCR (qRT-PCR) was performed using the StepOne System (Applied Biosystems) using the SYBR green Master Mix (Applied Biosystems). Primer sequences are shown in Supplementary Table S2.

Measurement of anti-collagen antibodies

Anti-mouse CII IgG1 and IgG2a antibodies were determined in serum using ELISA, as reported before [38]. Briefly, 96 wells plates were coated with 0.1 µg of mouse type II collagen (chondrex). Non-specific binding sites were blocked by a 5% solution of milk powder. Serial dilutions of mouse sera were added, followed

by incubation with peroxidase-labeled isotype-specific goat-anti-mouse-IgG antibodies and 5-aminosalicylic acid as substrate. Absorbance was measured at 450 nm.

SAA serum levels
Serum amyloid A in serum was measured with ELISA according to the manufacturer's (R&D systems) guidelines.

Histology
Total ankle joints were isolated and fixed in 4% formaldehyde for 4 days, thereafter decalcified in 5% formic acid and embedded in paraffin. Tissue sections of 7µm were stained using hematoxylin and eosin to study synovial inflammation, cartilage destruction, and bone erosion.

Statistics
Differences in the relative abundance of bacterial taxa between groups were evaluated using Mann-Whitney test. Differences in operational taxonomic units (OTUs) were evaluated using two-tailed Student's t-test. We corrected for multiple testing using the Benjamini and Hochberg procedure with false discovery rate (FDR) set at 10%, and differences with a p-value < 0.05 which passed the FDR test were considered statistically significant. Mann-Whitney U test was used to compare cell and cytokine levels between treatment groups. For arthritis scores, two-tailed Mann-Whitney U test was performed for area under the curve. Correlations were examined using Spearman's rank test. Statistical significance was indicated on figures as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Altered composition of intestinal microbiota marks the preclinical phase of CIA and precedes the development of arthritis
To examine whether perturbations of intestinal microbiota precede or rather result from the inflammatory arthritis, we analyzed the intestinal microbiota of naive DBA1/J mice before immunization with collagen type II (CII) and 21 days later prior to the booster injection using microbial 16S rRNA sequencing. Mice showed no macroscopic or histological signs of arthritis 21 days after immunization confirming the preclinical state (Supplementary Figure 1A). We did not observe any significant changes in the number of operation taxonomic units (OTUs), the rarefaction curves of Chao1 index of bacterial richness, and the phylogenetic distance whole tree, a diversity metric based on both the number of observed OTUs and their phylogenetic distance (data not shown). Therefore, induction of CIA did not affect the intestinal bacterial richness and diversity.

However, the preclinical, immune-priming phase of CIA was accompanied by marked compositional changes in intestinal microbiota at different taxonomic depths from phylum to genus. Analysis of the relative abundances at phylum level showed that the intestinal microbiota of naive mice is dominated by the phylum Bacteroidetes (Figure 1A-B). Comparison of the microbiota of naive vs. immunized mice revealed that phylum Firmicutes dominates the intestinal microbiota in the preclinical phase of arthritis, during which the relative abundance of Bacteroidetes decreases (Figure 1A-C). In addition, the relative abundance of Proteobacteria significantly increased after CIA immunization (Figure 1D).

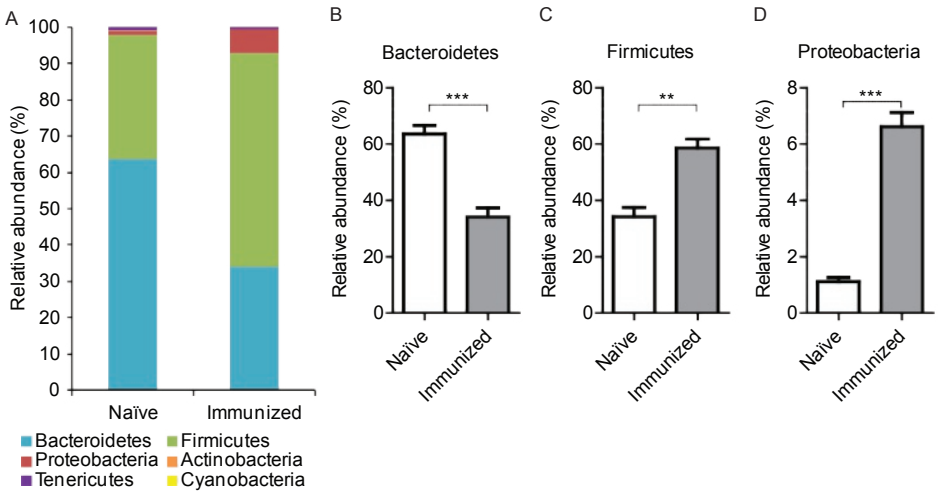


Figure 1. Altered composition of fecal microbiota after immunization with type II collagen. (A-D) 16S sequencing of fecal microbiota of naive DBA mice just before immunization with type II collagen compared with microbiota of mice 21 days post-immunization before the booster injection and arthritis development. Relative abundances of bacterial taxa on phylum level is shown (N=7 mice per group). Data are shown as mean + SEM. **p<0.01, ***p<0.001 by Mann-Whitney test followed by a correction for multiple testing using the Benjamini-Hochberg procedure.

At the family level, we found that the families S24-7, Bacteroidaceae and Paraprevotellaceae in the phylum Bacteroidetes were significantly reduced in the immune-priming phase of CIA (Figure 2A-D). Among Firmicutes, the families Lachnospiraceae and Ruminococcaceae were expanded while Lactobacillaceae and Erysipelotrichaceae were reduced during the preclinical phase of arthritis (Figure 2A, E-H). Furthermore, the family Desulfovibrionaceae in the phylum Proteobacteria was significantly increased in immunized compared with naive mice (Figure 2I). After correction for multiple testing using the Benjamini-Hochberg procedure, we observed a significant increase in the relative abundance of the genera *Oscillospira* and *Ruminococcus* (both in Ruminococcaceae family) and a significant decrease in *Bacteroides* (family Bacteroidaceae),

Prevotella (family Paraprevotellaceae), and *Lactobacillus* (family Lactobacillaceae) (Supplementary Figure 2). Only a few OTUs could be assigned at the species level, including two OTUs assigned to *Lactobacillus reuteri* that were significantly reduced, and two OTUs aligned to the species *Ruminococcus gnavus* that were significantly increased in preclinical phase of CIA (Supplementary Table 1). Altogether, our data strongly suggest that alterations of the intestinal microbiota occur in the preclinical, induction phase of inflammatory arthritis and likely precede the clinical manifestations of the disease.

Immunization with type II collagen results in increased serum SAA levels and local Th17 cell frequency in the preclinical phase of arthritis

As mentioned earlier, no signs of arthritis could be observed on macroscopic or histological level (Supplementary Figure 1A). Serum amyloid A (SAA) has been shown to be upregulated at early stages of inflammatory arthritis. Therefore, we measured SAA protein levels in serum along with the gene expression of SAA isoforms in the synovium of naive mice in comparison with immunized mice at day 21 post-immunization before the onset of arthritis. SAA levels in serum showed a significant increase 21 days after immunization before the onset of arthritis compared to naive condition (Supplementary Figure 1B). In addition, we observed a non-significant increase of SAA3 expression in the knee synovial tissue ($p=0.2345$). However, SAA1 and SAA2 were expressed at low levels and remained similar to the naive condition (Supplementary Figure 1C). In addition, we analyzed alterations of the T helper cell response in joint-draining lymph nodes in the pre-clinical phase of CIA. Flow cytometry analysis showed that the percentage of Th17 cells was significantly higher in immunized (preclinical) mice compared to naive mice (Supplementary Figure 1D). The percentages Th1 and Treg cells and the absolute numbers of Th1, Th17 and Treg cells were not affected by immunization (Supplementary Figure 1D and E). These data suggest that immune activation characterized by increase in SAA serum levels and local Th17 activation precedes the development of CIA.

Treatment with broad-spectrum antibiotics results in partial elimination and compositional shifts in the intestinal microbiota of CIA mice

GF condition and elimination of microbiota before the induction of arthritis affects the intestinal T cell balance and initiation of arthritis [26-28, 32]. To examine whether the gut microbiota modulate T cell responses and disease progression during established arthritis, we treated mice with ongoing CIA with broad-spectrum antibiotics (ABX) previously shown to alter the abundance and composition of intestinal bacteria in naive mice [31, 39]. To determine the effects of antibiotic-treatment during CIA on the intestinal microbiota, we analyzed bacterial 16S rRNA sequences of ABX-treated and control CIA mice. Antibiotic treatment strongly reduced the number of 16S sequence reads compared with

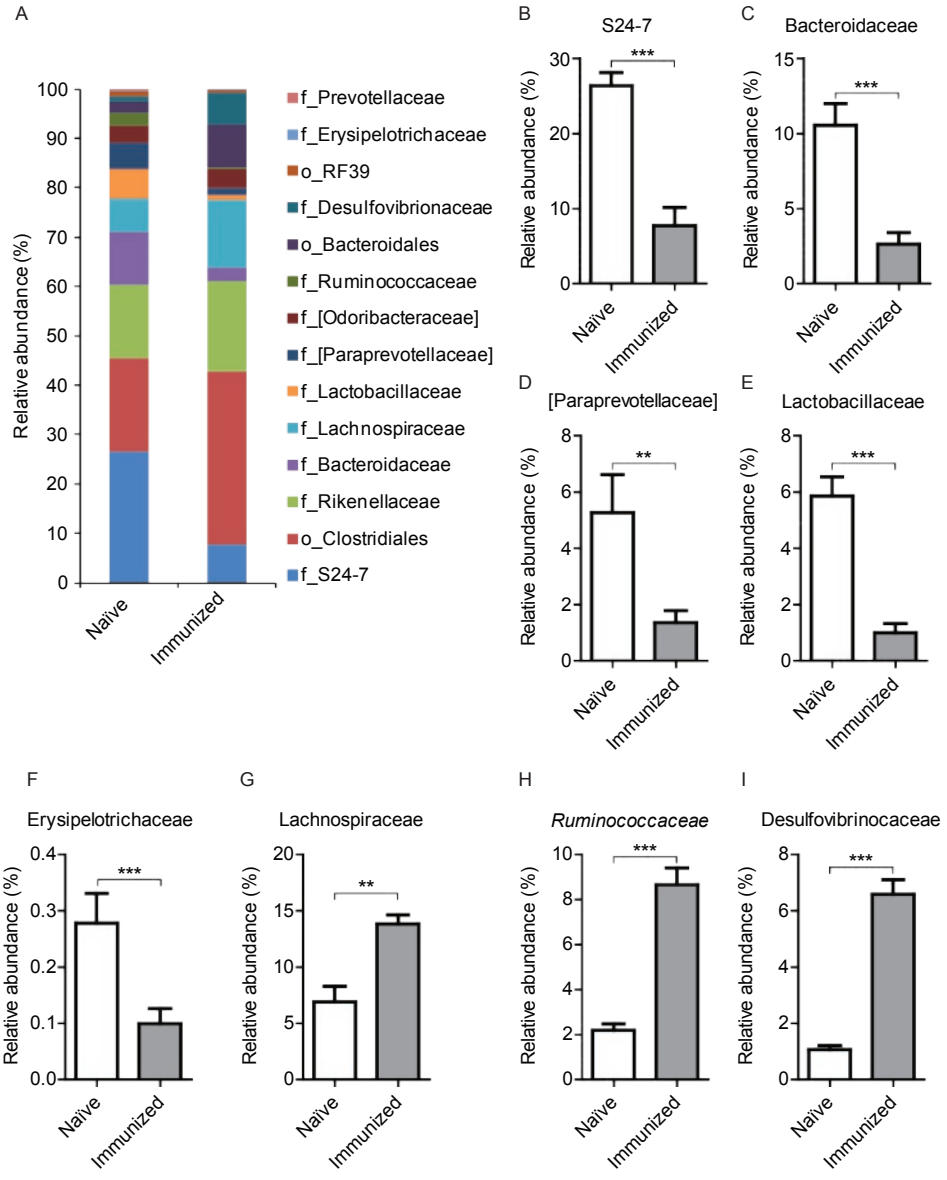


Figure 2. Intestinal microbiota undergoes marked changes in the preclinical phase of arthritis. (A) 16S sequencing of fecal microbiota of naive DBA mice just before immunization with type II collagen compared with microbiota of mice day 21 post-immunization and before the booster injection. Relative abundances on family level is shown, however part of the bacteria could not be classified further than order-level, taxonomic level is indicated, o=order and f=family. (B-I) Relative abundances of intestinal bacteria at family level. N=7 mice per group. Data are shown as mean + SEM. **p<0.01, ***p<0.001 by Mann-Whitney test followed by correction for multiple testing using the Benjamini-Hochberg procedure.

the control treatment (mean of 9002 ± 2167 versus 29392 ± 1140 reads, respectively; p= 0.004). In addition, antibiotic treatment resulted in reduced diversity and a marked compositional shift in the remaining intestinal bacterial communities of the CIA mice (Supplementary Figure 3). Specifically, almost all bacteria belonging to the phylum Bacteroidetes were eliminated (similar to response in humans [40]), and the majority of the bacteria that remained after antibiotic treatment belonged to the phylum Firmicutes (Supplementary Figure 3A). The relative abundances of intestinal bacterial taxa of the control and antibiotic-treated CIA mice at family and genus levels are shown in Supplementary Figure 3B and C.

Since SFB have been reported to trigger the development of autoimmunity in mice [28, 29, 41], we specifically examined the presence of SFB. We detected SFB (family Clostridiaceae; genus *Candidatus Arthromitus*) in the 16S sequences of only 2 out of 10 CIA mice before the ABX-treatment. After treatment SFB 16S sequences were detectable in 2 out of 5 untreated control mice but none of the ABX-treated mice. Using SFB-specific primers for qPCR [42], we could confirm the presence of SFB in all cages of CIA mice before the start of antibiotic treatment. At the end point, while we could still detect SFB in the untreated control group of CIA mice (Ct ≥ 32), SFB were not detectable in any of the ABX-treated CIA mice (data not shown).

Partial elimination of intestinal microbiota during ongoing CIA suppresses proinflammatory T helper cell subsets in intestinal lamina propria

It has been described that partial depletion of intestinal microbiota in naive mice results in reduced production of interferon γ (IFNγ) and IL-17 by mucosal $CD4^+$ T cells in small intestine lamina propria (LP) [39]. Therefore, we investigated whether depletion of intestinal microbiota during CIA affects the intestinal T helper cell balance. Flow cytometry analysis revealed a slight, non-significant reduction in the proportion of $TCR\beta^+CD4^+$ T helper cells in intestinal LP after ABX treatment (p=0.06, Figure 3A-B). Antibiotic treatment resulted in a specific reduction in the percentage of $TCR\beta^+CD4^+IL\text{-}17^+$ Th17 cells in LP of mice with CIA (p=0.004, Figure 3A and C), similar to previously published observations in naive mice [39]. In addition, a reduction in $TCR\beta^+CD4^+IFN\gamma^+$ Th1 cells was observed after ABX treatment; however, this effect was heterogeneous and did not reach statistical significance (p=0.1059, Figure 3A and C). In contrast, ABX treatment did not affect the proportion of $TCR\beta^+CD4^+Foxp3^+$ Treg cells in the intestinal LP of CIA mice (Figure 3C).

Next, we analyzed cytokine production by LP lymphocytes (LPLs) stimulated ex vivo with PMA and ionomycin. In accordance with the flow cytometry data, IL-17 production was significantly reduced in LPLs of ABX-treated mice compared with control mice (Figure 3D). IL-4 showed a trend towards reduction (p=0.0892; Supplementary Figure 4A), while the production of IFNγ and IL-10 remained

unaffected (p=0.2428 and p=0.9705, respectively; Figure 3E and Supplementary Figure 4B). Analysis of the gene expression showed that ABX treatment significantly reduced expression of IL-17A mRNA in terminal ileum (p= 0.008), a main site for microbiota-induced T cell modulation, whereas the expression IFNγ and IL-10 mRNA was not affected significantly (Supplementary Figure 4C). The expression of IL-17, IFNγ and IL-10 in colon tissue was not affected by antibiotic treatment (Supplementary Figure 4D) In addition, while unaffected in small intestine, the expression of the Treg-related transcription factor FoxP3 was significantly upregulated in the colon of ABX-treated mice (Supplementary Figure 4E). Altogether, these observations indicate that partial elimination of intestinal microbiota during established CIA modulates the mucosal T helper cell balance mainly presented by a suppression of Th17 cells.

Antibiotic treatment reduces intestinal SAA1 and SAA2 expression in CIA mice
The mechanism of microbiota-induced Th17 cell differentiation in intestinal LP was reported for SFB as model organisms. It was demonstrated that SFB-induced intestinal epithelial production of SAA1 and SAA2 in terminal ileum, stimulated by ILC3-derived IL-22, is required for the functional differentiation of Th17 cells and the production of IL-17 [30]. Therefore, we analyzed the expression of SAA1, SAA2 and SAA3 isoforms as well as IL-22 in terminal ileum. In line with the reduction of Th17 cell abundance in LP upon ABX treatment (Figure 3A and C), both SAA1 and SAA2 were markedly diminished in terminal ileum of ABX-treated CIA mice compared with the control CIA mice (Figure 3F). However, SAA3 expression was lower than SAA1 and SAA2, and showed a non-significant reduction (Figure 3F). Furthermore, while IL-22 was detectable in the majority of control CIA mice (15 out of 18), its expression was markedly attenuated in ABX-treated CIA mice and was detectable only in 2 out of 19 mice (Figure 3G). This suggests that reduced Th17 cell differentiation and IL-17 production in ABX-treated CIA mice may have resulted from lower IL-22-mediated induction of SAA1 and SAA2 due to the elimination of commensal microbiota.

Elimination of intestinal microbiota reduces the severity of established T cell-mediated experimental arthritis
We monitored the severity of arthritis in control and antibiotic-treated CIA mice. ABX treatment significantly attenuated the severity of established arthritis in CIA mice (Figure 4A). Interestingly, the abundance of Th17 cells in the intestinal LP of CIA mice showed a striking correlation with the severity of arthritis (r=0.666; p=0.001), while no correlation was found between the LP Th1 cells and arthritis (Figure 4B and C). This supports the presence of a potentially relevant link between the microbiota-induced intestinal Th17 cells and the severity of experimental arthritis. Importantly, the numbers of Th17 and Th1 cells in joint-draining pLN were significantly reduced in ABX-treated CIA mice compared

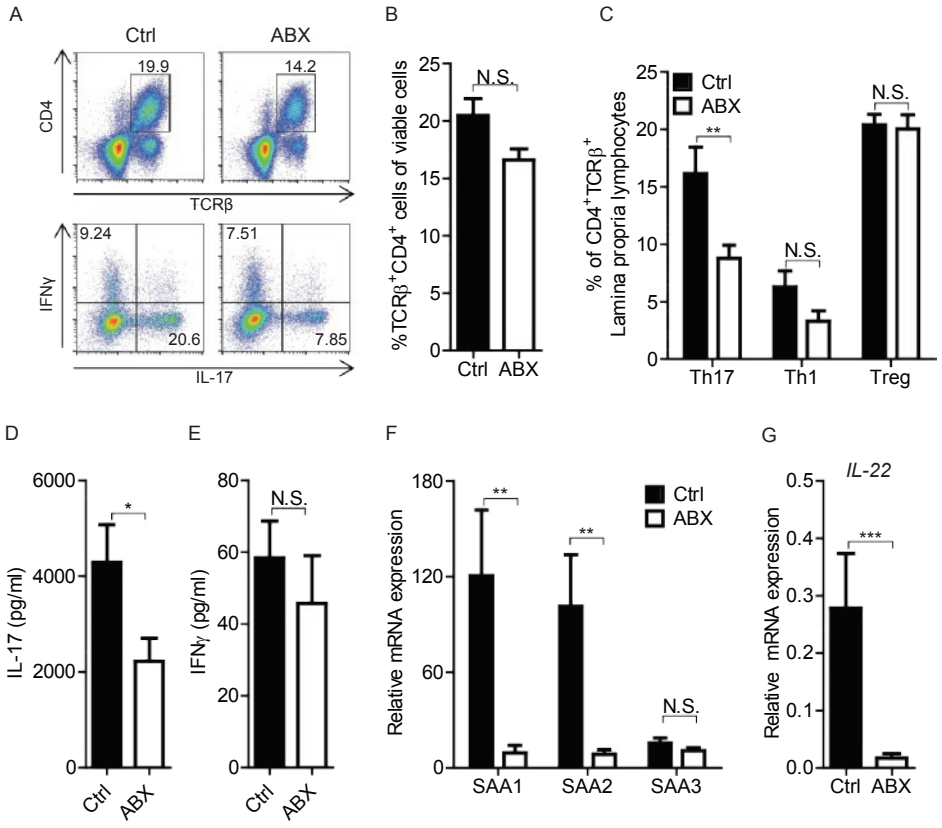


Figure 3. Treatment with broad-spectrum antibiotics during established CIA skews the intestinal T helper cell balance. (A) Representative flow cytometry plots showing the percentage of Th1 and Th17 cells per gate. (B) Percentage of $CD4^+TCR\beta^+$ Th cells of viable cells isolated from the intestinal lamina propria. (C) Percentage of IL-17^+ (Th17), IFNγ^+ (Th1) and $FoxP3^+$ (Treg) cells among $CD4^+TCR\beta^+$ cells isolated from intestinal lamina propria. (D,E) Production of IL-17 and IFNγ by lamina propria lymphocytes upon *ex vivo* stimulation with PMA and ionomycin for 5 hours, measured by Luminex cytokine array. (A-E) Cells were isolated from untreated control (Ctrl, n=12) and antibiotic-treated (ABX, n=10) mice at the end of the antibiotic treatment. (F-G) Gene expression of serum amyloid A (SAA) 1, 2 and 3 (F) and IL-22 (G) in terminal ileum of small intestine. Gene expression was measured by qPCR in tissues from Ctrl (n=18) and ABX (n=19) mice. Relative mRNA expression is shown as $2^{-\Delta Ct}$ *10000, corrected for GAPDH. Data are shown as mean + SEM. *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney test; N.S. not significant.

with control mice (Figure 4D). Accordingly, pLN cells from ABX-treated mice produced less IL-17 (p=0.0392) upon *ex vivo* stimulation with PMA and ionomycin (Figure 4E). The release of IFNγ in *ex vivo* LPL cultures was not significantly affected by the ABX treatment (p=0.3390, Figure 4F). Furthermore, the production of IL-10 and IL-4 remained unaffected (p=0.5179 and p=0.4417 respectively, data not shown). These observations suggest that partial elimination of intestinal microbiota after the onset of arthritis, i.e. during established CIA, attenuates the

disease and is associated with a parallel reduction in IL-17 production in joint-draining lymph nodes and intestinal LP. We further assessed the expression of the SAA isoforms in synovial tissue. In contrast to terminal ileum, SAA3 was the major isoform in the synovium and its expression was strongly decreased in antibiotic-treated CIA mice (p=0.0180, Figure 4G). Furthermore, reduced joint inflammation in antibiotic-treated CIA mice was accompanied by a significant suppression of SAA1 (p=0.0471) and a non-significant reduction of SAA2 (p=0.0771) mRNA expression in synovial tissue (Figure 4G).
Interestingly, while both LP and pLN Th17 cells were affected, we could not find any difference in serum levels of anti-mouse collagen type II auto-antibodies between the control and ABX-treated mice (Supplementary Figure 5). Therefore, the observed reduction in CIA disease severity by post-onset antibiotic treatment is not associated with a suppression of CII-specific antibody response.
In addition, elimination of intestinal microbiota by the ABX treatment was unable to modulate the severity of serum-transfer arthritis, a T cell-independent model [43, 44] induced by the transfer of arthritogenic K/BxN serum (Supplementary Figure 6). These data suggest that elimination of intestinal microbiota during T cell-mediated experimental arthritis can attenuate Th17-driven disease processes, whereas arthritis induced by the already generated arthritogenic antibodies does not depend on the intestinal microbiota.

Discussion

Several studies have shown that the composition of intestinal microbiota is perturbed in patients with inflammatory arthritis such as new-onset as well as chronic RA [5-7, 9, 45]. The development of autoimmunity in RA starts several years before the appearance of the clinical signs, a process that commensal microbiota may be able to promote [46-48]. Therefore, it is important to understand whether the observed perturbations in the intestinal microbiota precede the onset of inflammatory arthritis or are merely a consequence of disease. While this remains to be determined in humans at-risk of developing inflammatory arthritis, we here show that in experimental arthritis, marked changes in the intestinal microbiota occur in the preclinical immune-priming phase and precede the onset of inflammatory arthritis. A recent study showed that the composition of microbiota prior to arthritis onset differs between the CIA–susceptible and –resistant mice [49]. This study found that the family Lactobacillaceae was more abundant in CIA-susceptible mice, whereas Desulfovibrionaceae and Lachnospiraceae were more abundant in CIA–resistant mice [49]. Moreover, by comparing the fecal bacterial composition in the immunized mice before and after the onset of arthritis, it was shown that the families Bacteroidaceae, Lachnospiraceae and S24-7 significantly increased as arthritis initiated [49].

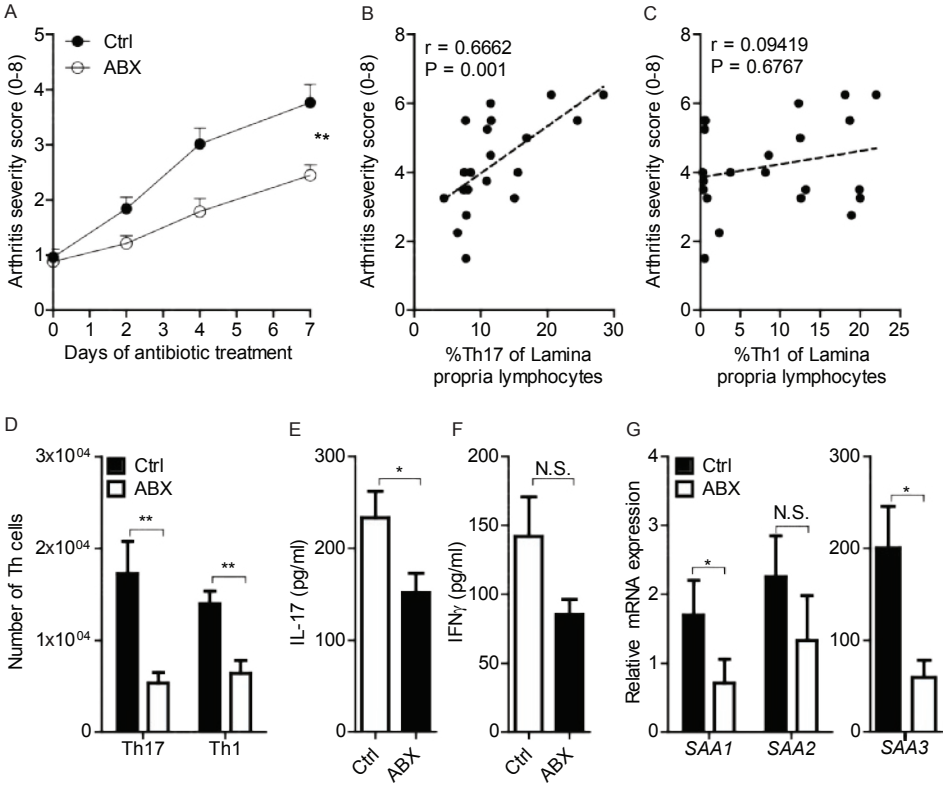


Figure 4. Elimination of intestinal microbiota reduces the severity of established T cell-mediated experimental arthritis. (A) Arthritis severity scores of untreated control (Ctrl) and antibiotic-treated (ABX) CIA mice; n=19 mice per group from two independent experiments. Severity was scored on a scale of 0-2 for each paw. **p<0.01 by two-tailed Mann-Whitney U test. (B,C) Correlation between the percentages of Th17 and Th1 cells in the intestinal lamina propria and arthritis severity scores of mice with CIA. The Spearman's correlation coefficient (r) and the p value are shown at the top left of the graphs. (D) Number of Th17 ($CD4^+TCR\beta^+$ $IL\text{-}17^+$) and Th1 ($CD4^+TCR\beta^+IFN\gamma^+$) cells isolated from popliteal lymph nodes (pLN) of Ctrl (n=11) and ABX-treated (n=10) CIA mice. (E,F) Concentration of IL-17 and IFNγ in culture supernatants of pLN cells from Ctrl (n=12) and (n=11) ABX mice *ex vivo* stimulated with PMA and ionomycin for 5 hours. (G) Gene expression of SAA1, SAA2 and SAA3 in synovial biopsies of Ctrl (n=12) and ABX (n=13) mice. Relative mRNA expression is shown as $2^{-\Delta Ct}$ *10000, corrected for GAPDH. (D-G) Data are shown as mean + SEM. *p<0.05, **p<0.01 by Mann-Whitney test.

Our studies focused on the preclinical phase of arthritis and show that this phase is characterized by decreased S24-7 and Bacteroidaceae and increased Ruminococcaceae, Lachnospiraceae and Desulfovibrinocaceae. Increased Lachnospiraceae has previously also been demonstrated in RA patients with varying disease duration [7]. Our data demonstrate that the increase in Lachnospiraceae occurs before the onset of arthritis and is induced by the immunization during the preclinical phase. Another study compared the microbiome of healthy non-immunized mice and mice with CIA.

This study found that Clostridiales, Lachnospiraceae and *Ruminoccocus gnavus* were more abundant in the mice with established CIA compared to the healthy mice [50]. Interestingly, we found these taxa to be increased in the preclinical phase and before the onset of arthritis. Altogether, these observations suggest that early perturbations of the intestinal microbiota during the immune-priming phase may contribute to the initiation of inflammatory arthritis.

We previously showed that treatment with Tobramycin resulted in a near-complete elimination of the genera *Helicobacter* and *Flexispira* (both belonging to the family Helicobacteraceae) and suppressed arthritis in IL-1Ra-deficient mice [51]. In addition, a strong and highly significant reduction in the genus *Clostridium* was observed, which suggests that these bacteria may promote intestinal Th17 cell differentiation and arthritis [51]. In the present study, we observed a strong and significant increase in the order Clostridiales after immunization with CII. However, a large part of the order Clostridiales could not be assigned to a higher taxonomic level and the exact effects on the genus *Clostridium* remain to be clarified.

Previous studies showed that *Prevotella copri* is expanded in patients with new-onset RA [6, 8]. In addition, it was shown that transfer of *Prevotella*-dominated microbiota of RA patients to SKG mice results in increased numbers of intestinal Th17 cells and exacerbates arthritis compared with mice receiving fecal microbiota from healthy controls [8]. Identification of the exact *Prevotella* species altered upon immunization in CIA mice was not possible using 16S rRNA gene sequencing due to inherently limited resolution of this technique. Furthermore, none of the observed OTUs related to *Prevotella* in our database could be assigned to *P. copri*. Therefore, we performed *P. copri*-specific qPCR using species specific primers [6] to detect *P. copri* with a higher sensitivity (primer sequences in Suppl. Table S2). While our positive control (DNA isolated from anaerobically grown *P. copri*) showed a positive signal on qPCR with a Ct value of 17.7 cycles, *P. copri* DNA was detected at very low levels (Ct value of >33 cycles) in fecal DNA of the mice and was not different between naive and immunized mice. Therefore, the abundance of *P. Copri* was not altered by CII immunization.

Loss of intestinal microbial diversity and richness coincides with autoimmune diseases such as diabetes, RA and PsA in patients [5, 6, 52]. Our data suggest that the preclinical phase of experimental arthritis is not accompanied by significant changes in the microbial diversity and richness. It is tempting to speculate that the reduced microbial diversity reported in human autoimmune disease may be a consequence of the ongoing inflammation at the time of sampling. While our study was able to identify robust changes in the intestinal microbiota that precede the onset of inflammatory arthritis, it is important to expand and strengthen such studies with shotgun sequencing coupled with transcriptomics and metabolomics approaches to understand the full functional capacity of the

microbiome during the preclinical phase of arthritis. More importantly, similar studies are warranted in humans at risk of developing autoimmune arthritis before the onset of inflammation to unravel the relevance of the microbiome in predisposition or the inductive phase of the disease.

Intestinal LP is a major source of Th17 cells in the body [29, 53]. Previous studies have shown that alterations of the gut commensal bacteria have strong effects on Th17 cell differentiation [29, 53]. Commensal microbiota have been shown to promote the initiation of spontaneous arthritis in IL-1Ra$^{-/-}$ mice as well as in K/BxN mice, which bear a transgenic auto-reactive T cell receptor [27, 28]. In non-transgenic animal models, treatment with a cocktail of vancomycin, neomycin and metronidazole before the induction of antigen-induced arthritis resulted in milder disease [32]. On the other hand, gut microbiota-induced IL-1 and IL-6 can trigger the respective cytokine receptors on B cells to promote differentiation of IL-10-producing regulatory B cells and restrict antigen-induced arthritis [32]. However, it is not clear whether modulation of intestinal microbiota after the onset of arthritis can still alter the T cell phenotype and modify the progression of arthritis.

In this study, we employed a widely-used model of inflammatory arthritis induced in non-transgenic mice to assess the role of intestinal microbiota during already established disease. We show here for the first time that elimination of intestinal microbiota during established CIA attenuates inflammatory arthritis. This is in line with earlier reports that oral antibiotic treatments with sulfasalazine and minocycline reduce RA disease activity in patients with disease duration of <1 year [54, 55]. Elimination of microbiota after the onset of arthritis resulted in suppression of intestinal SAA1, SAA2 and IL-22 expression and a specific reduction of LP Th17 cells accompanied with reduced Th17 cell abundance in joint-draining lymph nodes of the CIA mice. The potential of intestinal T cells to migrate to peripheral lymph nodes, as reported before, may explain these observations [56]. Our data also show for the first time a direct correlation between the abundance of LP Th17 cells and the severity of arthritis, which further supports a continued pro-inflammatory gut-joint axis during established arthritis.

Previous studies identified SFB as potent inducers of Th17 cell differentiation promoting arthritis development in the T cell-mediated K/BxN model [28, 29]. Elimination of SFB by ABX treatment in our study is consistent with reduced LP Th17 cells and suppressed expression of SAA1 and SAA2, which were recently reported to act on Th17 cells to further enhance their differentiation and effector function[30]. In line with the essential role of IL-22 in intestinal SAA production [30], we observed a significant reduction of intestinal IL-22 expression after antibiotic treatment. Our data suggest that the observed Th17 cell reduction upon antibiotic treatment in our study is at least partially due to the elimination of SFB, although the effect of other microbiota cannot be excluded.

Interestingly, although SAA1 and SAA2 were the most abundant SAA isoforms in the intestine, SAA3 appeared to be the prominent isoform in the target tissue, i.e. synovium, and its expression was similarly affected by the microbiota. While SAA1/SAA2 were shown to promote intestinal Th17 cells, it is not known if SAA has a similar effect on Th17 cells residing in the synovium. Several studies have shown the relevance of SAA as a marker of RA disease activity and its association with disease-relevant autoantibodies and acute phase proteins [57-60]. In addition, several RA therapies have been shown to influence circulating SAA levels during treatment [57-60]. Furthermore, SAA is also a good indicator of cardiovascular and renal involvement in patients with RA [61]. Besides being a valuable marker for disease activity, SAA has been shown to mediate inflammatory and angiogenic mechanisms, likely through TLR2 [62, 63]. Our data demonstrate that increased systemic level of SAA precedes the onset of arthritis and can be used as a disease marker even before its clinical onset.

In line with the essential role of IL-22 in intestinal SAA production [30], we observed a significant reduction of intestinal IL-22 expression after antibiotic treatment. IL-22 was shown to be highly expressed in synovium of RA patients and serum levels of IL-22 are significantly higher in RA patients compared with healthy controls and correlate with disease activity [64, 65]. In addition, elevated levels of IL-22 in early disease seem to predict erosive progression, suggesting that IL-22 has a role in the pathophysiology of RA [66]. In patients with PsA, IL-22-producing $CD4^+$ T cells were increased in peripheral blood as well; however, IL-22-positive cells could not be detected in synovial fluid and tissue of PsA patients [67]

Although our studies support suppression of intestinal and joint-adjacent Th17 differentiation as an underlying mechanism for attenuation of arthritis upon elimination of intestinal microbiota, involvement of microbiota-induced regulation of other immune cells is not excluded. In this regard, a recent study showed that continuous treatment with ampicillin and vancomycin limited follicular T helper (Tfh) cell differentiation and germinal center formation in K/BxN mice, resulting in reduced production of arthritogenic autoantibodies and less severe arthritis [68]. These effects appeared to be independent of IL-17, which was found to be dispensable for the disease development in this study, although an earlier study showed that IL-17 blockade could reduce anti-GPI antibody levels and suppress K/BxN arthritis [28, 68]. We observed no effect of ABX treatment on serum levels of anti-mouse collagen type II antibodies in our experiments, suggesting that during ongoing CIA, intestinal microbiota affect Th17 cell differentiation and arthritis without influencing the autoantibody response. Therefore, intestinal microbiota appear to influence different disease mechanisms depending on the disease phase and processes being studied. In addition, by mimicking the effector phase of the K/BxN arthritis using KRN serum-transfer model, we show that intestinal microbiota do not play an important

role in K/BxN arthritis anymore once the disease-inducing auto-antibodies have been developed (Supplementary Figure 5).
In agreement with our findings, IL-17 producing T cells can promote arthritis independent of their influence on antibody production, since transfer of IL-17-producing KRN transgenic T cells into a B-cell-deficient host can enhance arthritis in an IL-17-dependent manner [69]. In fact, Th17 cells contribute to several pro-inflammatory and tissue-destructive processes during inflammatory arthritis and may represent a relevant target to control the disease [12, 13]. The role of Th17 cells in arthritis is likely to expand beyond its prototypic cytokine IL-17A. The actual requirement of Th17 cells for the microbiota-induced aggravation of arthritis requires, however, further investigation.
It has been described that Th17 cells can transition into a stage in which they produce both IL-17 and IFNγ, after which they may lose their expression of IL-17 [70]. A recent study showed that these IFNγ-producing cells, called ex-Th17 cells or non-classical Th1 cells, are not constrained by regulatory T cells [71]. In addition, these cells were shown to accumulate in arthritic joint of arthritis patients [71], suggesting a role in the pathogenesis of RA. We analyzed the proportions of IL-17$^+$ IFNγ$^+$ double-positive TCRβ$^+$CD4$^+$ cells. We observed a non-significant reduction in the percentage, but not absolute number, of IL-17$^+$ IFNγ$^+$ TCRβ$^+$CD4$^+$ in intestinal lamina propria by antibiotic treatment (Mean ± SEM of control: 1.049% ± 0.2594; ABX: 0.4699% ± 0.1297). Furthermore, while there was no significant effect of antibiotic treatment on the percentage of IL-17$^+$ IFNγ$^+$ TCRβ$^+$CD4$^+$ cells in pLNs, we observed a non-significant reduction in the numbers of these cells (Ctrl: 537.9 ± 228.6 ; ABX: 240.6 ±76.38). We cannot exclude the possibility that the Th1 cells, which were significantly reduced in pLNs after ABX-treatment, also included exTh17 cells only producing IFNγ.
Multiple studies showed that Th17 levels are high in treatment-naive patients with early RA [72-74]. While elevated levels of Th17 cells have been found in RA and PsA patients, reports on the proportion of Th17 cells in patients with established RA are inconsistent [67, 72, 75-80]. Based on the higher efficacy of IL-17 blockade in PsA compared with RA, it is plausible that the PsA microbiome has a higher potential to induce mucosal Th17 and IL-17 responses. On the other hand, it is possible that stratification of patients with RA based on their gut microbiota and its Th17-inducing potential improves the efficacy of IL-17 inhibition in RA. A side-by-side comparison of matched cohorts of PsA and RA patients will be required to address the exact differences between RA and PsA microbiomes and their relevance for the efficacy of biologic treatments.
In summary, our study suggests that perturbations in the intestinal microbiota precede the onset of arthritis. In addition, we show that elimination of intestinal microbiota during established CIA reduces Th17 cell abundance in intestinal mucosa and joint-draining lymph nodes and attenuates arthritis without affecting autoantibody production. While our study does not advocate the use

treatment for RA or related inflammatory arthropathies, it supports the notion that inflammatory signals provided by the gut microbiota continue to promote arthritis after its onset. The striking correlation between the abundance of intestinal Th17 cells and the severity of arthritis supports this link. Understanding the exact mechanisms linking the intestinal T cell response with arthritis may help identifying novel therapeutic strategies for inflammatory arthritis.

References

1. Abdollahi-Roodsaz S, Abramson SB, Scher JU. The metabolic role of the gut microbiota in health and rheumatic disease: mechanisms and interventions. Nature reviews Rheumatology. 2016;12(8):446-55.
2. Scher JU, Littman DR, Abramson SB. Microbiome in Inflammatory Arthritis and Human Rheumatic Diseases. Arthritis & rheumatology (Hoboken, NJ). 2016;68(1):35-45.
3. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19.
4. Schett G, Coates LC, Ash ZR, Finzel S, Conaghan PG. Structural damage in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis: traditional views, novel insights gained from TNF blockade, and concepts for the future. Arthritis research & therapy. 2011;13 Suppl 1:S4.
5. Scher JU, Ubeda C, Artacho A, Attur M, Isaac S, Reddy SM, et al. Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease. Arthritis & rheumatology (Hoboken, NJ). 2015;67(1):128-39.
6. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife. 2013;2:e01202.
7. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med. 2015;21(8):895-905.
8. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T cells in the intestine. Arthritis & rheumatology (Hoboken, NJ). 2016.
9. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome medicine. 2016;8(1):43.
10. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75-84.
11. Kamada N, Nunez G. Regulation of the immune system by the resident intestinal bacteria. Gastroenterology. 2014;146(6):1477-88.
12. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. Nature reviews Rheumatology. 2015;11(7):415-29.
13. van den Berg WB, McInnes IB. Th17 cells and IL-17 a--focus on immunopathogenesis and immunotherapeutics. Semin Arthritis Rheum. 2013;43(2):158-70.
14. Avci AB, Feist E, Burmester GR. Targeting GM-CSF in rheumatoid arthritis. Clinical and experimental rheumatology. 2016;34(4 Suppl 98):39-44.
15. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. Nature reviews Rheumatology. 2016;12(1):37-48.
16. Tesmer LA, Lundy SK, Sarkar S, Fox DA. Th17 cells in human disease. Immunological reviews. 2008;223:87-113.
17. Mease PJ, Genovese MC, Greenwald MW, Ritchlin CT, Beaulieu AD, Deodhar A, et al. Brodalumab, an anti-IL17RA monoclonal antibody, in psoriatic arthritis. The New England journal of medicine. 2014;370(24):2295-306.
18. Mease PJ, McInnes IB, Kirkham B, Kavanaugh A, Rahman P, van der Heijde D, et al. Secukinumab Inhibition of Interleukin-17A in Patients with Psoriatic Arthritis. N Engl J Med. 2015;373(14):1329-39.
19. Genovese MC, Greenwald M, Cho CS, Berman A, Jin L, Cameron GS, et al. A phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. Arthritis & rheumatology (Hoboken, NJ). 2014;66(7):1693-704.

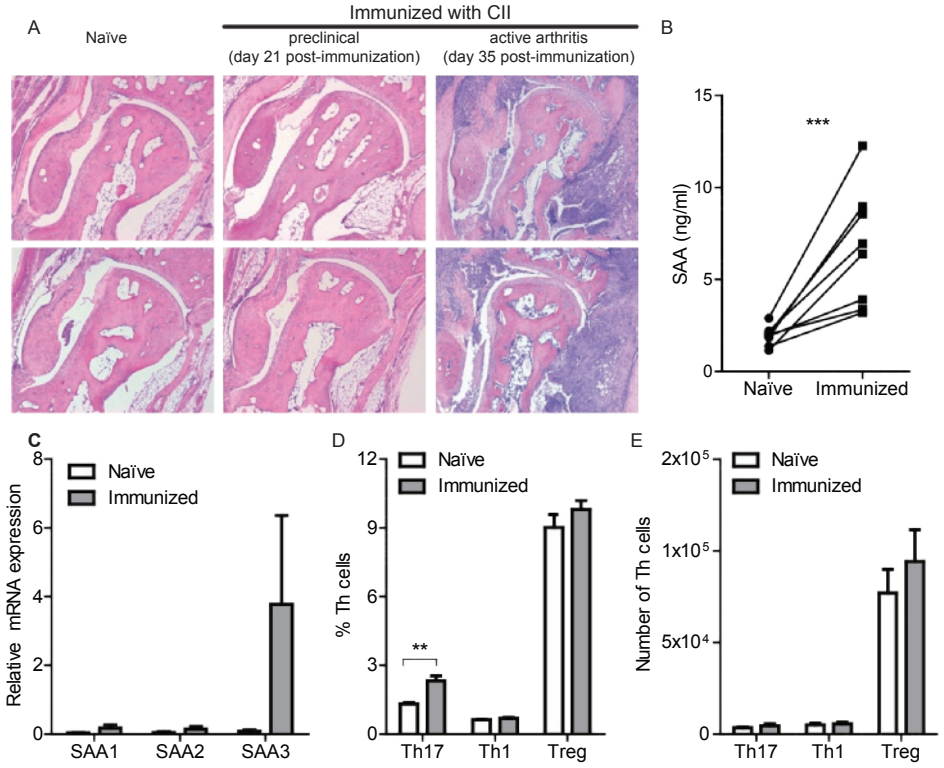
20. Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Aelion JA, et al. One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study. The Journal of rheumatology. 2014;41(3):414-21.
21. Burmester GR, Durez P, Shestakova G, Genovese MC, Schulze-Koops H, Li Y, et al. Association of HLA-DRB1 alleles with clinical responses to the anti-interleukin-17A monoclonal antibody secukinumab in active rheumatoid arthritis. Rheumatology (Oxford, England). 2016;55(1):49
22. Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova FE, et al. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. Am J Pathol. 2005;167(1):141-9.
23. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med. 2006;203(12):2673-82.
24. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest. 1999;103(9):1345-52.
25. Hot A, Miossec P. Effects of interleukin (IL)-17A and IL-17F in human rheumatoid arthritis synoviocytes. Annals of the rheumatic diseases. 2011;70(5):727-32.
26. Rehaume LM, Mondot S, Aguirre de Carcer D, Velasco J, Benham H, Hasnain SZ, et al. ZAP-70 genotype disrupts the relationship between microbiota and host, leading to spondyloarthritis and ileitis in SKG mice. Arthritis & rheumatology (Hoboken, NJ). 2014;66(10):2780-92.
27. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. J Clin Invest. 2008;118(1):205-16.
28. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
29. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485-98.
30. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. Cell. 2015;163(2):381-93.
31. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. Journal of immunology (Baltimore, Md : 1950). 2009;183(10):6041-50.
32. Rosser EC, Oleinika K, Tonon S, Doyle R, Bosma A, Carter NA, et al. Regulatory B cells are induced by gut microbiota-driven interleukin-1beta and interleukin-6 production. Nat Med. 2014;20(11):1334-9.
33. Marietta EV, Murray JA, Luckey DH, Jeraldo PR, Lamba A, Patel R, et al. Human Gut-Derived Prevotella histicola Suppresses Inflammatory Arthritis in Humanized Mice. Arthritis & rheumatology. 2016.
34. Joosten LA, Helsen MM, van de Loo FA, van den Berg WB. Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF alpha, anti-IL-1 alpha/beta, and IL-1Ra. Arthritis and rheumatism. 1996;39(5):797-809.
35. Scher JU, Joshua V, Artacho A, Abdollahi-Roodsaz S, Ockinger J, Kullberg S, et al. The lung microbiota in early rheumatoid arthritis and autoimmunity. Microbiome. 2016;4(1):60.
36. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. The ISME journal. 2012;6(3):610-8.
37. Abdollahi-Roodsaz S, Koenders MI, Walgreen B, Bolscher J, Helsen MM, van den Bersselaar LA, et al. Toll-like receptor 2 controls acute immune complex-driven arthritis in mice by regulating the inhibitory Fcgamma receptor IIB. Arthritis and rheumatism. 2013;65(10):2583-93.

38. Abdollahi-Roodsaz S, Joosten LA, Roelofs MF, Radstake TR, Matera G, Popa C, et al. Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. Arthritis and rheumatism. 2007;56(9):2957-67.
39. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, et al. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. 2010;3(2):148-58.
40. Isaac S, Scher JU, Djukovic A, Jimenez N, Littman DR, Abramson SB, et al. Short- and long-term effects of oral vancomycin on the human intestinal microbiota. The Journal of antimicrobial chemotherapy. 2017;72(1):128-36.
41. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4615-22.
42. Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N, et al. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. Infect Immun. 2008;76(3):907-15.
43. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity. 1999;10(4):451-61.
44. Kyburz D, Corr M. The KRN mouse model of inflammatory arthritis. Springer Semin Immunopathol. 2003;25(1):79-90.
45. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine. Arthritis & rheumatology (Hoboken, NJ). 2016;68(11):2646-61.
46. van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, et al. Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis and rheumatism. 2011;63(11):3226-33.
47. Deane KD, O'Donnell CI, Hueber W, Majka DS, Lazar AA, Derber LA, et al. The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner. Arthritis and rheumatism. 2010;62(11):3161-72.
48. van der Woude D, Rantapaa-Dahlqvist S, Ioan-Facsinay A, Onnekink C, Schwarte CM, Verpoort KN, et al. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. Annals of the rheumatic diseases. 2010;69(8):1554-61.
49. Liu X, Zeng B, Zhang J, Li W, Mou F, Wang H, et al. Role of the Gut Microbiome in Modulating Arthritis Progression in Mice. Scientific reports. 2016;6:30594.
50. Ben-Amram H, Bashi T, Werbner N, Neuman H, Fridkin M, Blank M, et al. Tuftsin-Phosphorylcholine Maintains Normal Gut Microbiota in Collagen Induced Arthritic Mice. Frontiers in microbiology. 2017;8:1222.
51. Rogier R, Ederveen THA, Boekhorst J, Wopereis H, Scher JU, Manasson J, et al. Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis. Microbiome. 2017;5(1):63.
52. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, et al. Toward defining the autoimmune microbiome for type 1 diabetes. The ISME journal. 2011;5(1):82-91.
53. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009;31(4):677-89.
54. Hannonen P, Mottonen T, Hakola M, Oka M. Sulfasalazine in early rheumatoid arthritis. A 48-week double-blind, prospective, placebo-controlled study. Arthritis and rheumatism. 1993;36(11):1501-9.
55. Stone M, Fortin PR, Pacheco-Tena C, Inman RD. Should tetracycline treatment be used more extensively for rheumatoid arthritis? Metaanalysis demonstrates clinical benefit with reduction in disease activity. The Journal of rheumatology. 2003;30(10):2112-22.

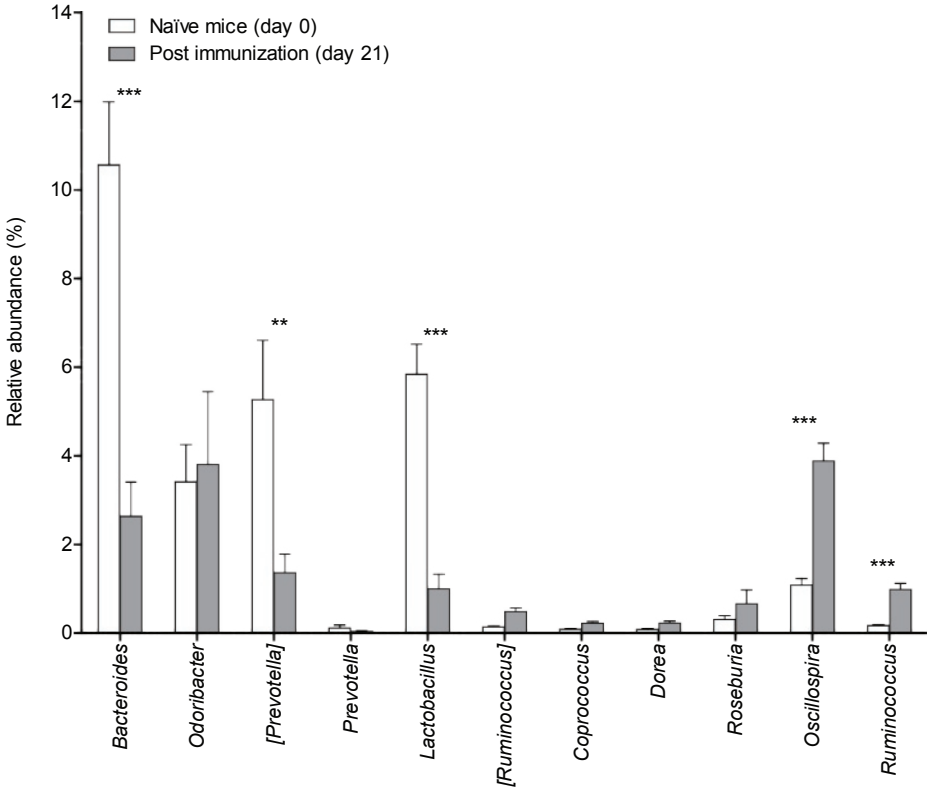
56. Morton AM, Sefik E, Upadhyay R, Weissleder R, Benoist C, Mathis D. Endoscopic photoconversion reveals unexpectedly broad leukocyte trafficking to and from the gut. Proc Natl Acad Sci U S A. 2014;111(18):6696-701.
57. Hwang YG, Balasubramani GK, Metes ID, Levesque MC, Bridges SL, Jr., Moreland LW. Differential response of serum amyloid A to different therapies in early rheumatoid arthritis and its potential value as a disease activity biomarker. Arthritis research & therapy. 2016;18(1):108.
58. Gabay C, McInnes IB, Kavanaugh A, Tuckwell K, Klearman M, Pulley J, et al. Comparison of lipid and lipid-associated cardiovascular risk marker changes after treatment with tocilizumab or adalimumab in patients with rheumatoid arthritis. Annals of the rheumatic diseases. 2016;75(10):1806-12.
59. Migita K, Izumi Y, Jiuchi Y, Kozuru H, Kawahara C, Izumi M, et al. Effects of Janus kinase inhibitor tofacitinib on circulating serum amyloid A and interleukin-6 during treatment for rheumatoid arthritis. Clinical and experimental immunology. 2014;175(2):208-14.
60. Shen C, Sun XG, Liu N, Mu Y, Hong CC, Wei W, et al. Increased serum amyloid A and its association with autoantibodies, acute phase reactants and disease activity in patients with rheumatoid arthritis. Molecular medicine reports. 2015;11(2):1528-34.
61. Targonska-Stepniak B, Majdan M. Serum amyloid A as a marker of persistent inflammation and an indicator of cardiovascular and renal involvement in patients with rheumatoid arthritis. Mediators of inflammation. 2014;2014:793628.
62. Hong C, Shen C, Ding H, Huang S, Mu Y, Su H, et al. An involvement of SR-B1 mediated p38 MAPK signaling pathway in serum amyloid A-induced angiogenesis in rheumatoid arthritis. Molecular immunology. 2015;66(2):340-5.
63. Connolly M, Rooney PR, McGarry T, Maratha AX, McCormick J, Miggin SM, et al. Acute serum amyloid A is an endogenous TLR2 ligand that mediates inflammatory and angiogenic mechanisms. Annals of the rheumatic diseases. 2016;75(7):1392-8.
64. da Rocha LF, Jr., Duarte AL, Dantas AT, Mariz HA, Pitta Ida R, Galdino SL, et al. Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity. The Journal of rheumatology. 2012;39(7):1320-5.
65. Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, et al. Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. Arthritis and rheumatism. 2005;52(4):1037-46.
66. Leipe J, Schramm MA, Grunke M, Baeuerle M, Dechant C, Nigg AP, et al. Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis. Annals of the rheumatic diseases. 2011;70(8):1453-7.
67. Benham H, Norris P, Goodall J, Wechalekar MD, FitzGerald O, Szentpetery A, et al. Th17 and Th22 cells in psoriatic arthritis and psoriasis. Arthritis research & therapy. 2013;15(5):R136.
68. Block KE, Zheng Z, Dent AL, Kee BL, Huang H. Gut Microbiota Regulates K/BxN Autoimmune Arthritis through Follicular Helper T but Not Th17 Cells. Journal of immunology (Baltimore, Md : 1950). 2016;196(4):1550-7.
69. Jacobs JP, Wu HJ, Benoist C, Mathis D. IL-17-producing T cells can augment autoantibody-induced arthritis. Proc Natl Acad Sci U S A. 2009;106(51):21789-94.
70. Shi G, Cox CA, Vistica BP, Tan C, Wawrousek EF, Gery I. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. Journal of immunology (Baltimore, Md : 1950). 2008;181(10):7205-13.
71. Basdeo SA, Cluxton D, Sulaimani J, Moran B, Canavan M, Orr C, et al. Ex-Th17 (Nonclassical Th1) Cells Are Functionally Distinct from Classical Th1 and Th17 Cells and Are Not Constrained by Regulatory T Cells. Journal of immunology (Baltimore, Md : 1950). 2017;198(6):2249-59.
72. Leipe J, Grunke M, Dechant C, Reindl C, Kerzendorf U, Schulze-Koops H, et al. Role of Th17 cells in human autoimmune arthritis. Arthritis and rheumatism. 2010;62(10):2876-85.

73. Colin EM, Asmawidjaja PS, van Hamburg JP, Mus AM, van Driel M, Hazes JM, et al. 1,25-dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. Arthritis and rheumatism. 2010;62(1):132-42.
74. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis and rheumatism. 2011;63(1):73-83.
75. van Hamburg JP, Corneth OB, Paulissen SM, Davelaar N, Asmawidjaja PS, Mus AM, et al. IL-17/Th17 mediated synovial inflammation is IL-22 independent. Annals of the rheumatic diseases. 2013;72(10):1700-7.
76. Yamada H, Nakashima Y, Okazaki K, Mawatari T, Fukushi JI, Kaibara N, et al. Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. Annals of the rheumatic diseases. 2008;67(9):1299-304.
77. Miao J, Geng J, Zhang K, Li X, Li Q, Li C, et al. Frequencies of circulating IL-17-producing CD4+CD161+ T cells and CD4+CD161+ T cells correlate with disease activity in rheumatoid arthritis. Modern rheumatology. 2014;24(2):265-570.
78. Chalan P, Kroesen BJ, van der Geest KS, Huitema MG, Abdulahad WH, Bijzet J, et al. Circulating CD4+CD161+ T lymphocytes are increased in seropositive arthralgia patients but decreased in patients with newly diagnosed rheumatoid arthritis. PloS one. 2013;8(11):e79370.
79. Jandus C, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P. Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. Arthritis and rheumatism. 2008;58(8):2307-17.
80. Raychaudhuri SK, Saxena A, Raychaudhuri SP. Role of IL-17 in the pathogenesis of psoriatic arthritis and axial spondyloarthritis. Clinical rheumatology. 2015;34(6):1019-23.

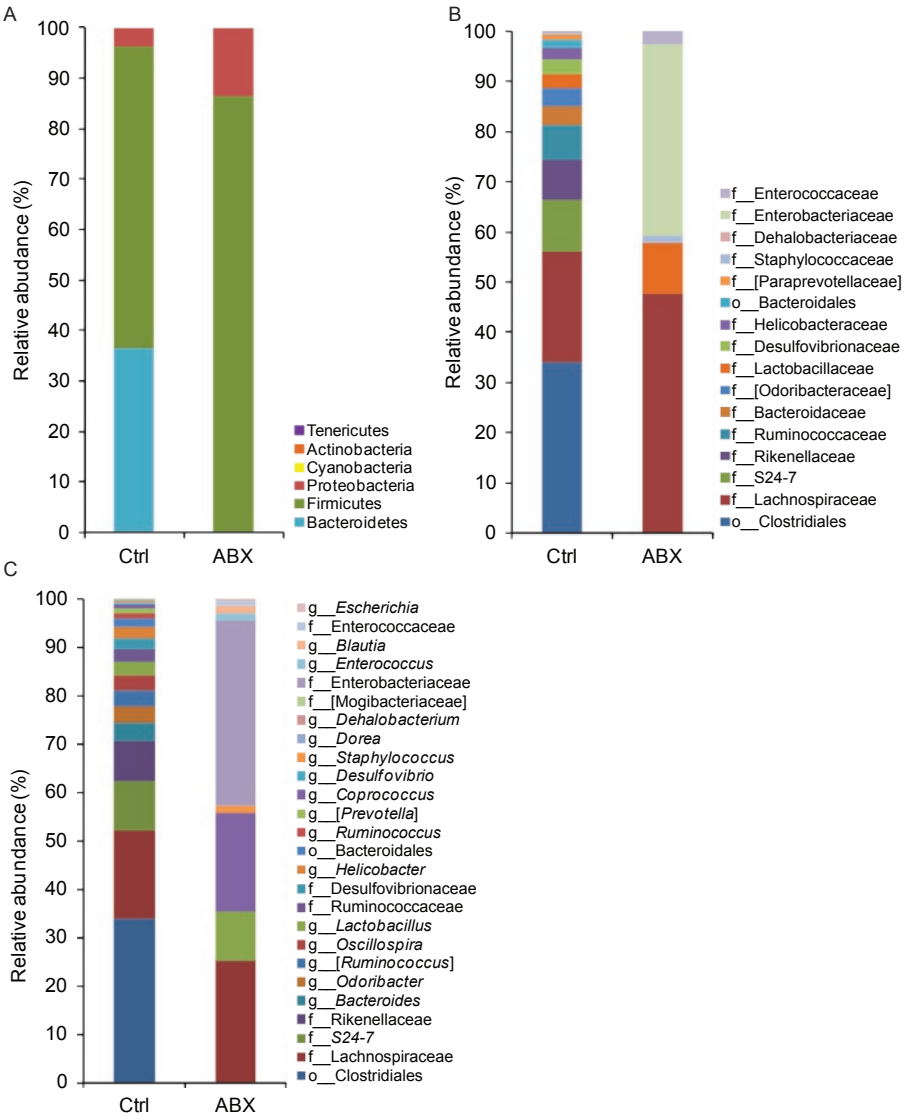
Supplementary material



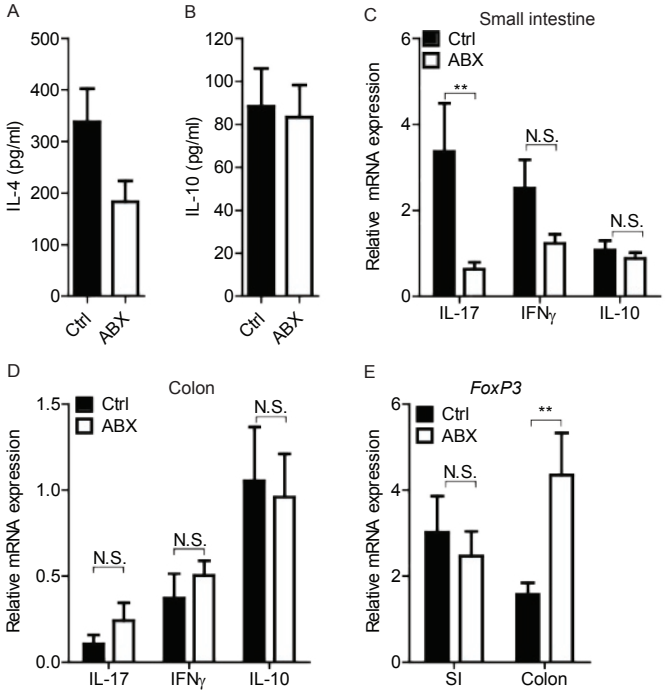
Supplementary Figure 1. Serum SAA levels significantly increased in preclinical phase of collagen induced arthritis. (A) Representative pictures of ankle joints of naive mice and mice 21 (preclinical) or 35 days (active arthritis) post-immunization with collagen type II (CII). (B) SAA serum levels of naive and mice 21 days after immunization (n=8). (C) Gene expression of SAA1, SAA2 and SAA3 in synovial biopsies of naive (n=9) and immunized mice (n=8). Relative mRNA expression is shown as $2^{-\Delta Ct}$ *10000, corrected for GAPDH. (D-E) Percentage (D) or total number (E) of Th17 ($CD4^+TCR\beta^+$ $IL\text{-}17^+$) and Th1 ($CD4^+TCR\beta^+IFN\gamma^+$) cells isolated from popliteal lymph nodes (pLN) of naive mice (n=6) and mice 21 days after immunization (n=6) with CII. Data are shown as mean + SEM. **p<0.01, ***p<0.001 by Mann-Whitney test.



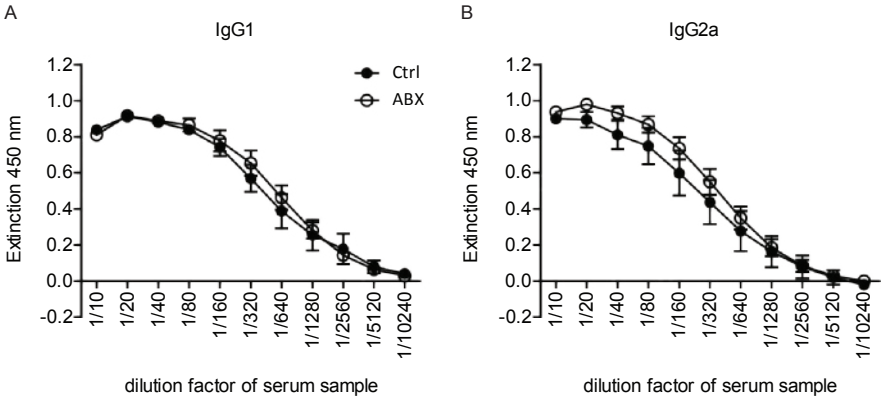
Supplementary Figure 2. Preclinical phase of collagen induced arthritis is marked by a shift in intestinal microbiota. Relative abundance of different genera present in intestinal microbiota of naive mice (day 0) and mice 21 days after immunization (day 21). Genera with a relative abundance of > 0.1% in either of the two groups are shown. Data is shown as mean +SEM of n=7 mice per group. **p<0.01, ***p<0.001, by Mann-Whitney test followed by a correction for multiple testing using the Benjamini-Hochberg procedure.



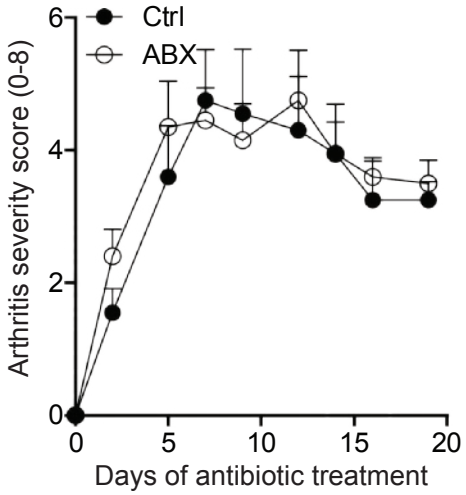
Supplementary Figure 3. Treatment with broad-spectrum antibiotics causes shift in intestinal bacterial community composition. (A-C) Intestinal bacterial composition at the phylum (A), family (B) and genus (C) level of untreated control (Ctrl, n=5) and antibiotic-treated (ABX, n=6) mice. Feces were collected 3 days after discontinuation of treatment which was the end-point of the experiment. o=order, f= family and g=genus.



Supplementary Figure 4. Treatment with broad-spectrum antibiotics results in shift in intestinal Th cell subsets. (A-B) Production of IL-4 and IL-10 by lamina propria mononuclear cells of untreated control (Ctrl, n=12) and antibiotic-treated (ABX, n=10) mice with CIA upon *ex vivo* stimulation with PMA and ionomycin for 5 hours, measured by Luminex cytokine array. (C-D) Gene expression of IL-17, IFN and IL-10 in small intestine (C) and colon (D)of Ctrl (n=18) and ABX (n=19) mice, as measured by PCR. (E) Gene expression of FoxP3 in small intestine (SI) and colon of Ctrl and ABX mice. Relative mRNA expression is shown as $2^{-\Delta Ct}$ *10000, corrected for GAPDH. Data are shown as mean + SEM, Mann-Whitney test **p<0.01. N.S. not significant.



Supplementary Figure 5. Serum levels of anti-mouse collagen type II antibodies not affected by antibiotic treatment. Serum levels of IgG1 (A) and IgG2a (B) anti-type II collagen antibodies of untreated control (Ctrl) and antibiotic-treated (ABX) mice (n=7 mice per group) measured by ELISA.



Supplementary Figure 6. Antibiotic treatment does not affect severity of serum-transfer arthritis. Macroscopic arthritis severity scores of serum-transfer arthritis (0-2 per paw) of untreated control (Ctrl) and antibiotic-treated (ABX) mice. Data is shown as mean + SEM, of n=5 mice per group.

Supplementary Table 1. Differentially abundant operational taxonomic units (OTUs) in microbiota of naive and immunized mice. Relative abundance (percentage) of differentially present OTUs in intestinal microbiota of naive (day 0) and collagen II-immunized (day 21 post-immunization) mice. P-values were calculated using a Student's t-test followed by a correction for multiple testing using the Benjamini-Hochberg procedure; n=7 mice per group.

	Day 0		Day 21		
Taxonomic assignment	Mean	SEM	Mean	SEM	P-value
o_Bacteroidales	0.106	0.025	0.005	0.002	0.0070
o_Bacteroidales	2.342	0.656	0.149	0.042	0.0155
g_*Odoribacter*	0.207	0.062	0.046	0.030	0.0451
g_[*Prevotella*]	0.125	0.030	0.023	0.012	0.0130
g_[*Prevotella*]	4.566	1.186	1.145	0.350	0.0276
g_*Bacteroides*	0.152	0.026	0.026	0.006	0.0026
g_*Bacteroides*	0.181	0.031	0.042	0.015	0.0033
g_*Bacteroides*	1.063	0.194	0.179	0.040	0.0035
g_*Bacteroides*	0.437	0.079	0.086	0.021	0.0038
g_*Bacteroides*	0.220	0.040	0.050	0.020	0.0042
g_*Bacteroides*	2.727	0.631	0.403	0.075	0.0101
g_*Bacteroides*	0.114	0.029	0.013	0.004	0.0136
g_*Bacteroides*	0.110	0.030	0.018	0.008	0.0200
g_*Bacteroides*	3.535	0.812	1.133	0.409	0.0272
f_Rikenellaceae	0.029	0.006	0.107	0.014	0.0009
f_Rikenellaceae	0.396	0.058	0.099	0.024	0.0014
f_Rikenellaceae	0.542	0.087	0.180	0.058	0.0057
f_Rikenellaceae	1.909	0.126	0.966	0.233	0.0060
f_Rikenellaceae	0.184	0.040	0.034	0.009	0.0094
f_Rikenellaceae	0.062	0.008	0.133	0.021	0.0143
f_S24-7	0.554	0.030	0.106	0.022	0.0000
f_S24-7	0.679	0.055	0.166	0.032	0.0000
f_S24-7	1.390	0.134	0.161	0.048	0.0000
f_S24-7	0.116	0.012	0.013	0.004	0.0000
f_S24-7	0.833	0.102	0.089	0.028	0.0002
f_S24-7	1.048	0.163	0.150	0.056	0.0011
f_S24-7	0.181	0.025	0.048	0.016	0.0011
f_S24-7	3.760	0.565	0.742	0.260	0.0011
f_S24-7	0.422	0.066	0.057	0.015	0.0012
f_S24-7	0.179	0.030	0.020	0.006	0.0015
f_S24-7	0.265	0.046	0.039	0.014	0.0021
f_S24-7	3.031	0.333	1.109	0.374	0.0024
f_S24-7	0.150	0.026	0.030	0.010	0.0026
f_S24-7	0.121	0.023	0.010	0.004	0.0027
f_S24-7	0.738	0.129	0.126	0.031	0.0028
f_S24-7	2.835	0.619	0.286	0.121	0.0059
f_S24-7	0.221	0.030	0.086	0.030	0.0085

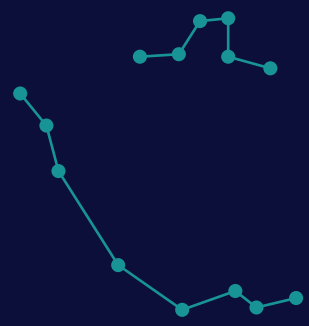
	Day 0		Day 21		
Taxonomic assignment	Mean	SEM	Mean	SEM	P-value
f_S24-7	0.369	0.084	0.051	0.019	0.0086
f_S24-7	0.226	0.058	0.012	0.005	0.0102
f_S24-7	0.948	0.078	0.447	0.138	0.0109
f_S24-7	0.213	0.050	0.033	0.012	0.0111
f_S24-7	0.640	0.195	0.028	0.008	0.0202
f_S24-7	0.126	0.040	0.005	0.002	0.0220
f_S24-7	0.306	0.085	0.055	0.012	0.0254
g_*Lactobacillus*	0.136	0.020	0.013	0.006	0.0005
g_*Lactobacillus*	0.290	0.061	0.077	0.024	0.0122
g_*Lactobacillus*	2.548	0.819	0.177	0.069	0.0275
g_*Lactobacillus*	1.088	0.280	0.278	0.102	0.0278
s_*reuteri*	0.100	0.013	0.029	0.012	0.0016
s_*reuteri*	0.653	0.120	0.180	0.055	0.0065
o_Clostridiales	0.364	0.104	0.969	0.089	0.0009
o_Clostridiales	0.057	0.007	0.194	0.027	0.0019
o_Clostridiales	0.013	0.004	0.109	0.026	0.0091
o_Clostridiales	0.006	0.004	0.125	0.032	0.0093
o_Clostridiales	0.128	0.012	0.279	0.042	0.0103
o_Clostridiales	0.053	0.021	0.273	0.063	0.0121
o_Clostridiales	0.189	0.114	1.033	0.275	0.0220
o_Clostridiales	0.300	0.089	0.058	0.021	0.0345
o_Clostridiales	0.040	0.010	0.227	0.071	0.0392
o_Clostridiales	0.098	0.031	0.507	0.159	0.0425
f_Lachnospiraceae	0.023	0.008	0.163	0.036	0.0075
f_Lachnospiraceae	0.104	0.030	0.287	0.048	0.0087
f_Lachnospiraceae	0.053	0.014	0.142	0.027	0.0177
f_Lachnospiraceae	0.151	0.038	0.391	0.091	0.0415
s_*gnavus*	0.078	0.015	0.157	0.020	0.0092
s_*gnavus*	0.056	0.008	0.233	0.067	0.0381
f_Ruminococcaceae	0.042	0.010	0.209	0.021	0.0001
f_Ruminococcaceae	0.035	0.010	0.192	0.026	0.0006
f_Ruminococcaceae	0.161	0.028	0.411	0.043	0.0006
f_Ruminococcaceae	0.014	0.006	0.191	0.043	0.0058
f_Ruminococcaceae	0.102	0.028	0.383	0.074	0.0082
f_Ruminococcaceae	0.153	0.032	0.831	0.208	0.0170
g_*Oscillospira*	0.040	0.012	0.238	0.032	0.0004
g_*Oscillospira*	0.096	0.014	0.369	0.044	0.0005
g_*Oscillospira*	0.043	0.013	0.181	0.039	0.0112
g_*Oscillospira*	0.091	0.035	0.511	0.125	0.0146
g_*Oscillospira*	0.027	0.006	0.108	0.024	0.0154
g_*Oscillospira*	0.068	0.016	0.175	0.042	0.0462
g_*Ruminococcus*	0.010	0.003	0.308	0.113	0.0380
f_Desulfovibrionaceae	0.062	0.008	0.293	0.019	0.0000
f_Desulfovibrionaceae	0.861	0.121	5.159	0.371	0.0000
f_Desulfovibrionaceae	0.010	0.003	0.121	0.014	0.0001
f_Desulfovibrionaceae	0.024	0.004	0.167	0.021	0.0003

Supplementary table 2. Primer sequences used for qRT-PCR.

Gene	Forward	Reverse
GAPDH	5’-GGCAAATTCAACGGCACA-3’	5’-GTTAGTGGGGTCTCGCTCTG-3’
IL-17A	5’-CAGGACGCGCAAACATGA-3’	5’-GCAACAGCATCAGAGACACAGAT-3’
IFNγ	5’-TCTTCTTGGATATCTGGAGGAACTG-3’	5’-AGAGATAATCTGGCTCTGCAGGAT-3’
Foxp3	5’-AGGAGAAGCTGGGAGCTATGC-3’	5’-GGTGGCTACGATTGCAGCAA-3’
SAA1	5’-TGCTGAGAAAATCAGTGATGGAA-3’	5’-GGTCAGCAATGGTGTCCTCAT-3’
SAA2	5’-GCTGACCAGGAAGCCAACA-3’	5’-GCAGTCCAGGAGGTCTGTAGTAATT-3’
SAA3	5’-GCAGCACGAGCAGGATGA-3’	5’-TCCCAGGATCAAGATGCAAAG-3’
IL-22	5’-GGTGCCTTTCCTGACCAAAC-3’	5’-CGTCACCGCTGATGTGACA-3’
UNI 16S	5’-ACTCCTACGGGAGGCAGCAGT-3’	5’-ATTACCGCGGCTGCTGGC-3’
P.Copri	5’-CCGGACTCCTGCCCCTGCAA-3’	5’-GTTGCGCCAGGCACTGCGAT-3’

Chapter 5

Microbiota-dependent involvement of Th17 cells in murine models of inflammatory arthritis



Chapter 5

Microbiota-dependent involvement of Th17 cells in murine models of inflammatory arthritis

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Abstract

Objective
Intestinal microbiota are associated with the development of inflammatory arthritis. The aim of this study was to dissect intestinal mucosal immune responses in the preclinical phase of arthritis and determine whether the presence of Th17 cells, beyond involvement of the cytokine interleukin-17 (IL-17), is required for arthritis development, and whether the involvement of Th17 cells in arthritis depends on the composition of the host microbiota.

Methods
Mucosal T cell production of IL-17, Interferon-γ, tumor necrosis factor α (TNFα), IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF) was analyzed by flow cytometry and Luminex assay before arthritis onset in mice immunized to develop collagen-induced arthritis (CIA). Pathogenic features of arthritis in mice with CIA and mice with antigen-induced arthritis were compared between Th17-deficient (*CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$) and Th17-sufficient (*CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$) mice. In addition, the impact of intestinal microbiota on the Th17 cell dependence of CIA was assessed.

Results
Lamina propria CD4 T cells were activated before the onset of arthritis in mice with CIA, with marked upregulation of several cytokines including IL-17A, TNFα and GM-CSF. *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ mice showed a specific reduction in mucosal levels of IL-17-producing CD8 T cells. However, total levels of IL-17A, mostly produced by γδ T cells and neutrophils, were unaffected. The severity of arthritis was significantly reduced in Th17 cell-deficient mice, suggesting that Th17 cells have additional, IL-17A-independent roles in inflammatory arthritis. Accordingly, antigen-stimulated T cells from Th17 cell-deficient mice produced less IL-17A, IL-17F and GM-CSF. Importantly, the dependence of CIA on the involvement of Th17 cells was mitigated in the presence of an alternative microbiome.

Conclusion
These data from murine models suggest that activation of mucosal immunity precedes the development of arthritis, and also that Th17 cells have a microbiota-dependent role in arthritis. Therefore, a microbiome-guided stratification of patients might improve the efficacy of Th17-targeted therapies.

Introduction

Intestinal microbiota are associated with several autoimmune diseases including rheumatoid arthritis (RA). Mucosal surfaces such as periodontal, lung and intestinal tissues are a proposed site of immune activation and breach of tolerance in RA [1]. However, the extent and nature of the mucosal immune activation during preclinical arthritis are unclear. Several independent studies report perturbed diversity and composition of commensal microbiota in patients with RA compared with healthy individuals [2-5]. However, the relationship between dysbiosis and RA pathogenesis is not fully understood.

The microbiome profoundly affects the balance between inflammatory $CD4^+$ T helper 1 (Th1) and Th17 cells and protective regulatory T (Treg) cells at mucosal surfaces and systemically [6]. Specific taxa such as segmented filamentous bacteria (SFB), enhance differentiation of Th17 cells in small intestine lamina propria (SI-LP) [7, 8]. SFB colonization exacerbates spontaneous arthritis in IL-1 receptor antagonist deficient and K/BxN mice [9, 10]. In addition, in SKG mice with increased thymic selection of auto-reactive T cells [11], colonization of *Prevotella copri*, a commensal species associated with new-onset RA [2, 3], increased SI-LP Th17 cell abundance and enhanced arthritis upon co-exposure to zymosan [3]. Although these studies showed that Th17-inducing intestinal microbiota exacerbate arthritis, it is not known whether the involvement of these microbiota-induced Th17 cells is required for the pahtogenesis of arthritis. Inhibition of either IL-17A or its receptor ameliorates arthritis in mice [12-15]. Although IL-17 blockade resulted in clinically relevant American College of Rheumatology 50 (ACR50) response in about 20% RA patients, convincing efficacy in the overall population was not observed [16-18]. Importantly, Th17 cells produce other proinflammatory mediators such as IL-17F, IL-22, TNFα, and GM-CSF, which have IL-17A-independent pro-inflammatory roles [13, 19-21]. Therefore, the role of Th17 cells in RA likely extends beyond IL-17A production. Additionally, previous studies in animal models and clinical trials used IL-17-deficient mice or IL-17 blocking antibodies. Because several cell types other than Th17 cells produce IL-17 [13, 22], the specific contribution of Th17 cells to arthritis pathogenesis unclear.

Enrichment of Th17 cells *in vivo* strongly depends on the presence and composition of intestinal microbiota [7, 8, 23]. Accordingly, the intestinal mucosa is a major anatomic compartment of Th17 cells [7, 8] and the involvement of Th17 cells in arthritis may be highly dependent on the composition of the host microbiota. Our aim was to determine the role of Th17 cells in arthritis pathogenesis and investigate whether the involvement of Th17 cells in arthritis depends on the microbiota present in the gut prior to disease development. In addition, we hypothesized that mucosal immune activation occurs in the preclinical phase and precedes the onset of arthritis.

Materials and methods

Mice

Th17-deficient mice (*CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$) on a C57BL/6 background were generated by crossing B6(Cg)-Rorc$^{tm3Litt/J}$ with B6.Cg-Tg(Cd4-cre) mice (Jackson Laboratories, stock #008771 and #022071) [24, 25]. *CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$ mice littermates were used as wild-type (WT) control mice. Experimental groups consisted of randomized sex- and age-matched *Cre*$^{+}$ and *Cre*$^{-}$ co-housed littermates. Mice were housed in individually ventilated cages and provided autoclaved food and water *ad libitum*.

Antibiotic treatments and reconstitution with Jackson microbiota

Age- and sex-matched groups of *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ and *CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$ mice received a cocktail of metronidazole, neomycin trisulfate, ampicillin sodium salt, vancomycin, and sucrose added to drinking water for 1 week. Microbiota was reconstituted by oral gavage of 200 µl aqueous suspension of SFB-free feces from Jackson mice 24 hours after ceasing antibiotics. SFB-free status was confirmed by qPCR as reported previously [10, 26].

Isolation of lamina propria cells

Mesenteric fat and Peyer's were removed from the small intestine and colon. Tissue was incubated with 5 mM EDTA to remove epithelial cells, and subsequently digested with 1 mg/ml collagenase-D and 10 µg/ml DNAse I . LP lymphocytes were harvested at the interphase of a 40:80% Percoll gradient and utilized as described.

Cell cultures and cytokine measurements

1-2 x 105 cells/well (as indicated in figure legends) SI-LP or LN cells were cultured in round-bottom 96 well plates. Supernatants were collected from PMA (50 ng/ml) and ionomycin (1 µg/ml) stimulated cells after 6 hours and from collagen (50 µg/mL) stimulated cells after 2 days. Cytokine levels were measured by Luminex using mouse cytokine group 1 and 3 magnetic bead kits (Bio-Rad) according to manufacturer instructions.

Flow cytometry

Prior to flow cytometry staining, cells were restimulated with PMA (50 ng/ml; Sigma), ionomycin, and Brefeldin A for 4 hours. Staining protocols and reagents are described in Supplementary methods and Supplementary Table 1. Cells were fixed in 2% PFA and stored at 4 C until acquisition with an LSRII flow cytometer. Analysis was performed in FlowJo.

Fluorescence-activated cell sorting
Splenocytes were stained with surface markers as described, then resuspended in T cell media and sorted with a FACS Aria II via the following parameters: TCRβ$^+$, viability dye negative cells were positively selected, followed by additional positive selection on CD4 and CD8 single positive cells.

Induction of antigen-induced arthritis (AIA)
To induce AIA, mice were treated with 200 µg mBSA in saline intraarticularly and 250 ng IL-1β in saline subcutaneously in the footpad, with additional IL-1β treatments at 24 and 48 hours [27, 28]. Mice were sacrificed on day 7, during the peak of the inflammatory response [27, 28].

Induction of collagen-induced arthritis (CIA)
CIA was induced via two intradermal immunizations with 100 µL of an emulsion consisting of a 1:1 ratio of chicken type II collagen (4 mg/mL in 10 mM acetic acid) and complete Freund's adjuvant (CFA), based on previously published protocols optimized for the Bl/6 background [29, 30]. CFA was prepared by adding 5 mg desiccated *M. tuberculosis* H37RA (Difco) per 1 mL incomplete Freund's adjuvant. Primary immunization was administered in the tail base at 10-12 weeks of age. Mice received a booster in the lower back at day 21 and were monitored for clinical signs of arthritis. All mice developed mild arthritis.

Assessment of arthritis
Arthritis severity was scored blindly as described previously [31]. For histology, ankle joints were isolated and fixed in 4% formaldehyde for 4 days, thereafter decalcified in 5% formic acid and embedded in paraffin. 7µm tissue sections of were stained using Haematoxylin & Eosin or Safranin O. Synovial inflammation, chondrocyte death, proteoglycan depletion, and erosion of hyaline cartilage were scored on a scale from 0-3 in a blinded manner [31].

DNA qPCR and 16S sequencing
Mouse genomic DNA was extracted from sorted CD4$^+$ and CD8$^+$ splenocytes with a QIAamp DNA mini kit (Qiagen) according to manufacturer instructions. Fecal pellets were collected and stored at -20 °C. DNA extraction was performed with the PowerLyzer DNA isolation kit (MO BIO laboratories) according to manufacturer instructions. qPCR was performed on the StepOne System (Applied Biosystems) using KAPA SYBR FAST Master Mix (KAPA Biosystems). Sequencing methodology is extensively described in the Supplementary methods. Primers are listed in Supplementary Table 2.

Statistical analysis
Measures are expressed as mean ± SEM. Statistical significance between two

groups was tested via Mann-Whitney U test or Student's T test, as indicated. Analysis of three or more groups was performed using Kruskal-Wallis test with an uncorrected Dunn's test. Arthritis scores were compared using repeated measures ANOVA with uncorrected Fisher's LSD test. Statistical analysis was performed using GraphPad Prism 7.02.

Results

Characterization of mucosal immune activation preceding the onset of arthritis

Modulation of the intestinal immune response by commensal microbiota affects the inflammation paterns and immunopathologic features at distal anatomic sites, including the joints [9, 10, 32, 33]. To investigate mucosal immune activation during preclinical arthritis, we assessed intestinal $CD4^+$ T cell cytokine responses before arthritis onset in WT mice 21 days after a single immunization with CII. We found that immunization of the mice with CII induced a robust T cell response in the SI lamina propria, with significantly increased percentages of both Th1 and Th17 cells (Figures 1A and B; Supplementary Figure 1A for gating strategy). In addition, $CD4^+$ T cell expression of TNFα, GM-CSF and IL-22 was upregulated in the SI lamina propria after immunization (Figures 1A and B). The numbers of LP $CD4^+$ T cells expressing inflammatory cytokines were increased upon immunization with CII (Supplementary Figure 2A), although not all analytes showed statistical significance. The measurement of cytokines in culture supernatants confirmed significantly increased SI lamina propria prodduction of IL-17 and IL-22 (Supplementary Figure 2B). Furthermore, we observed higher frequencies of $CD4^+$ T cells that expressed IL-17A, IL-22, and GM-CSF in the mesenteric lymph nodes (MLNs) of immunized mice. T cell IFNγ and TNFα production remained unaffected (Figure 1C, Supplementary Figure 1B). In the Peyer's patches (PP), percentages of TNFα, IL-22, and GM-CSF-producing $CD4^+$ T cells were increased in immunized mice, with no difference in the number of Th1 or Th17 cells (Supplementary Figure 1C). We observed no difference in $CD4^+$ T cell cytokine production in colonic lamina propria of naive mice compared wuth immunized mice (data not shown), suggesting that mucosal immune activation in response to CII immunization is restricted to the SI lamina propria.

Because Th17 cells produce multiple cytokines [20, 22], we examined whether CII immunization specifically altered the production of these cytokines by Th17 cells. SI lamina propria and MLN Th17 cells from immunized mice exhibited significantly higher coexpression of IL-22 compared with naive mice (Figure 1D). In addition, Th17 cell coexpression of IL-22 and GM-CSF was increased in the PP of immunized mice compared with naive mice (Supplementary Figure 1D). These data suggest that activation of intestinal $CD4^+$ T cells and increased production of mucosal Th1 and Th17 cells precedes the development of arthritis in mice.

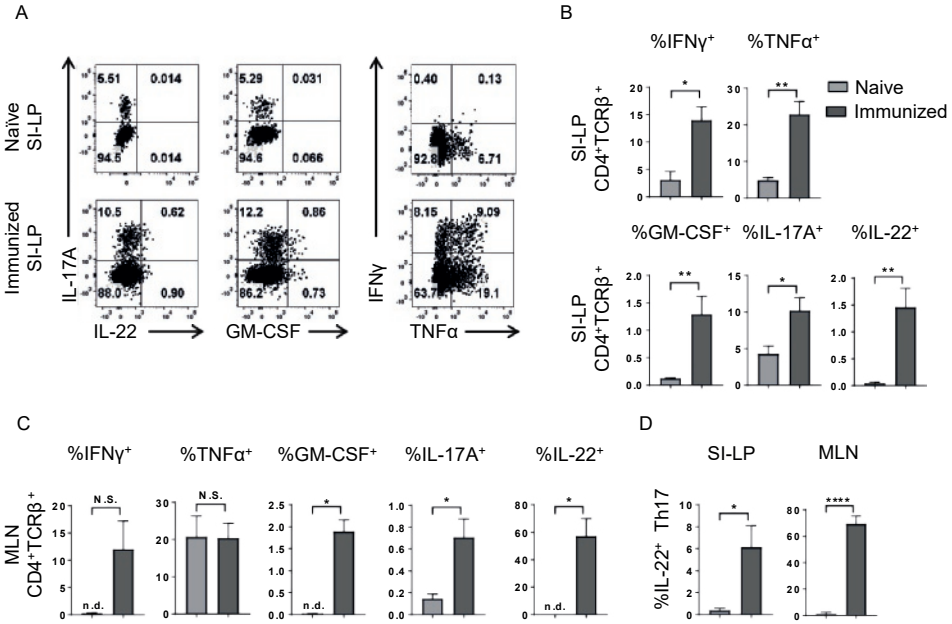


Figure 1. Intestinal mucosal CD4⁺ T cells are activated during the preclinical phase of arthritis and exhibit Th1 and Th17 phenotypes. Small intestine lamina propria (SI-LP) and mesenteric lymph node (MLN) lymphocytes from naive and single-immunized (non-arthritic) wild-type mice were isolated, restimulated with PMA and ionomycin, and stained for intracellular flow cytometry. Cells were gated as shown in Supplementary Figure 1A and cytokine production by CD4⁺ T cells was analyzed. (A) Representative FACS plots show CD4⁺ T cell production of IL-17A, IL-22, GM-CSF, IFNγ and TNFα in the SI-LP of naive and immunized mice. (B and C) Production of IFNγ, TNFα, GM-CSF, IL-17A and IL-22 by CD4⁺ T cells was determined in the SI-LP (B) and MLN (C) of naive and immunized mice. (D) Percentages of Th17 cells that coexpressed IL-22 in the SI-LP and MLNs of naive and immunized mice are shown. Results are mean ± SEM in cells from 4 mice per group, from a representative experiment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001, by Student't t-test. N.S. (not significant).

Specific reduction of Th17 cells but unaffected IL-17 production by other cellular sources in Th17 cell-deficient mice

Results from previous studies using IL-17 neutralizing antibodies or IL-17-deficient mice suggested that IL-17 has a role in the pathogenesis of arthritis [14, 15, 34-37]. However, because IL-17 is produced by multiple cell types other than Th17 cells, such as neutrophils and mast cells [13, 38, 39], the role of, and requirement for Th17 cells specifically has not been established. Notably, IL-17 deficiency protects against innate cell-mediated K/BxN serum-transfer arthritis [14, 15, 40, 41]. To directly examine the role of Th17 cells in the development of arthritis, we utilized conditional Th17-deficient mice. Th17 ablation in these mice is achieved through a CD4-specific Cre recombinase-induced deletion of a floxed *Rorc* allele, which prevents te expression of retinoic acid receptor-related orphan nuclear receptor γt (RORγt) and thus differentiation of naive CD4 T cells into Th17 cells [42]. *CD4-Cre$^{+/-}$STAT3$^{flox/flox}$* mice, which is anothher type of

Th17-deficient mouse strain, were not utilized in our studies because production of the Th1 and T follicular helper cell subsets is impaired in these mice [43,44].
To confirm that *CD4-Cre^{+}Rorc$^{flox/flox}$* mice had effective and specific deletion of IL-17 in CD4^{+} T cells, we compared T helper cell differentiation between *CD4-Cre^{+}Rorc$^{flox/flox}$* mice and control *CD4-Cre^{-}Rorc$^{flox/flox}$* littermates. We observed that the SI lamina propria, PP and MLNs of *CD4-Cre^{+}Rorc$^{flox/flox}$* mice had decreased percentages of CD4^{+} T cells compared with *CD4-Cre^{-}Rorc$^{flox/flox}$* control mice (Figure 2A and data not shown). Furthermore, the percentages and absolute numbers of Th17 cells were significantly reduced in the SI lamina propria (Figure 2B) and PP (Figure 2C) of Cre^{+} mice compared with Cre^{-} littermates. Th17 cells were negligible in the MLNs from both genotypes (data not shown). In contrast to Th17 cells, the percentages and absolute number of IFNγ- and TNFα-producing CD4^{+} T cells were not affected in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice (data not shown).
To assess proliferation and apoptosis of Th17 cells, we measured the expression of the proliferation marker Ki-67 on total live CD4^{-} cells, CD4^{+} IL-17^{-} cells, and Th17 cells by flow cytometry. We observed a significant reduction in the levels of Ki-67^{+} Th17 cells in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice. There was no difference in Ki-67 expression on CD4^{-} and non-Th17 CD4^{+} T cells between genotypes (Figure 3A). Therefore, these findings suggest that the *CD4-Cre^{+}Rorc$^{flox/flox}$* genotype leads to reduced proliferation in Th17 cells, but has no effect on other CD4^{+} T cells or non-CD4 cells. Furthermore, the numbers of annexin V^{+} apoptotic cells were increased among CD4^{+} T cells (both Th17 and non-Th17) in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice (Figure 3B). These data suggest that loss of Th17 cells in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice is a reflection of reduced proliferation and increased apoptosis of Th17 cells.
Because CD4-Cre is expressed at the CD4/CD8 double-positive stage of T cell development, Cre recombinase deletes floxed sequences in mature CD4 and CD8 T cells. To determine the effect of *CD4-Cre^{+}Rorc$^{flox/flox}$* genotype on CD8 T cells, we assessed deletion of the *Rorc* gene in genomic DNA of sorted TCRβ^{+} CD4^{+} CD8^{-} T cells and TCRβ^{+} CD4^{-} CD8^{+} T cells (purity of each subset >99%) via real-time PCR. As expected, the relative genomic presence of *Rorc* was significantly deleted in CD4^{+} T cells (P=0.0065). Expression of *Rorc* was also significantly depleted in CD8^{+} T cell genomic DNA from *CD4-Cre^{+}Rorc$^{flox/flox}$* mice, although the difference between genotypes did not reach statistical significance (P=0.23), presumably due to variation (Supplementary Figure 3A). On average, TCRβ^{+} CD4^{+} CD8^{-} and TCRβ^{+} CD4^{-} CD8^{+} cells sustained 25.8% and 26.7% of their genomic *Rorc*, respectively (Supplementary Figure 3B). To determine whether IL-17 production by other cells was altered in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice, we examined IL-17 expression in neutrophils, mast cells, and γδ T cells. Among SI lamina propria TCRβ-negative CD11b^{+} cells, the proportion of CD117^{+} mast cells was a mean ± SEM 0.34±0.15%. However, IL-17 expression was not detected in mast cells from naive mice of either genotype. These observations were confirmed in the

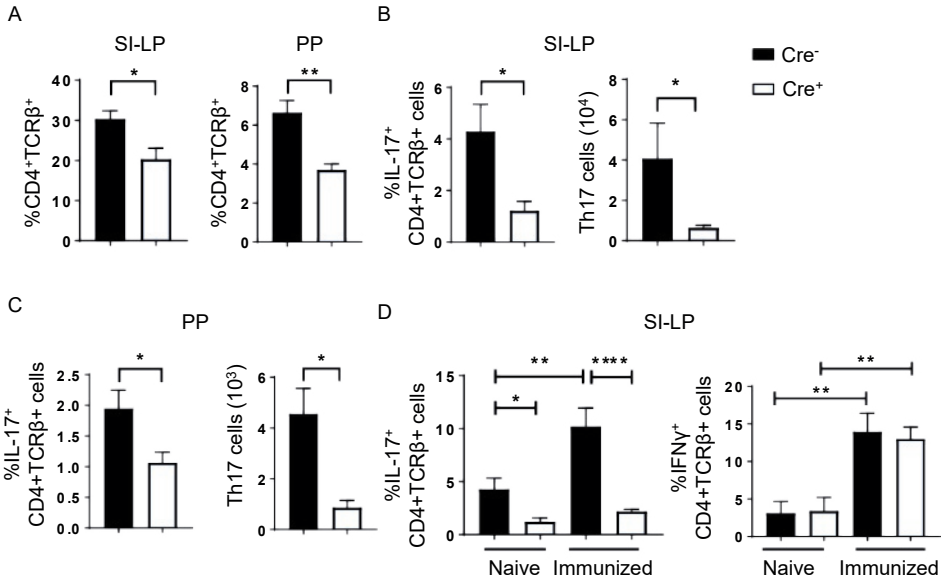


Figure 2. *CD4-Cre⁺ Rorc$^{flox/flox}$* mice exhibit specific Th17 cell deficiency under naive and immunized conditions. SI-LP and PP lymphocytes from 11-14-week-old male *CD4-Cre⁺ Rorc$^{flox/flox}$* and *CD4-Cre⁻ Rorc$^{flox/flox}$* mice were isolated and counted. Cells were then stimulated with PMA and ionomycin and stained for intracellular flow cytometry. (A-C) *Cre⁺* and *Cre⁻* mice were compared for percentages of CD4⁺ T cell in the SI-LP and PP (A), and for percentages of IL-17+ T cells within the live CD4⁺TCRβ⁺ gate and for absolutes number of IL-17A⁺ CD4⁺ T cells in the SI-LP (B) and PP (C). (D) Percentages of IL-17A⁺ and IFNγ⁺ cells within the live CD4⁺TCRβ⁺ T cell population in the SI-LP were compared between naive and immunized *Cre⁺* and *Cre⁻* mice. Results are mean ± SEM in cells from 4 mice per group, from a representative experiment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001, by student's t-test or by analysis of variance with Fisher's least significant difference test. See Figure 1 for other definitions.

colonic lamina propria, MLNs and PP (data not shown). Therefore, it can be concluded that mast cells are not a major cellular source of the mucosal IL-17 under naive conditions.

Analysis of CD11b+ cells expressing the surface marker Ly-6G, which is exclusively expressed on neutrophils, showed that a proportion of lamina propria neutrophils coexpressed Rorγt and IL-17. While the percentage of IL-17⁺ RORγt⁺ neutrophils was similar between *CD4-Cre⁺Rorc$^{flox/flox}$* mice, there was a non-significant increase in the percentage of IL-17⁺ and IL-17/RORγt double-positive neutrophils in *CD4-Cre⁺Rorc$^{flox/flox}$* mice (Figure 4A). Furthermore, the numbers of IL-17⁺ TCR γδ⁺ T cells were non-significantly increased in *CD4-Cre⁺Rorc$^{flox/flox}$* mice (P=0.26; Figure 4B). Therefore, these findings suggest that neutrophils and γδ T cells may compensate for the reduced CD4 and CD8 T cell-derived production of IL-17 in the lamina propria of *CD4-Cre⁺Rorc$^{flox/flox}$* mice. Since cytokine production is relatively low in naive mice, we sought to confirm specific Th17 deficiency in in antigen-experienced Cre⁺ mice. We euthanized naive mice and CII-immunized

CD4-Cre^{-}*Rorc*$^{flox/flox}$ mice and CD4-Cre^{+}*Rorc*$^{flox/flox}$ littermates and measured the proportions of Th1 and Th17 cells by flow cytometry. We found that IL-17 expression was up-regulated only in *Cre*$^{-}$ mice upon immunization (Figure 2D). As was observed in naice mice, the abundance of Th17 cells was significantly reduced in immunized *Cre*$^{+}$ mice compared with immunized *Cre*$^{-}$ mice (Figure 2D). In contrast, the numbers of intestinal Th1 cells were similary increased in both *Cre*$^{+}$ and *Cre*$^{-}$ mice upon immunization (Figure 2D). These data confirm that the production of Th17 cells, but not Th1 cells, is impaired in CD4-Cre^{+}*Rorc*$^{flox/flox}$ mice.

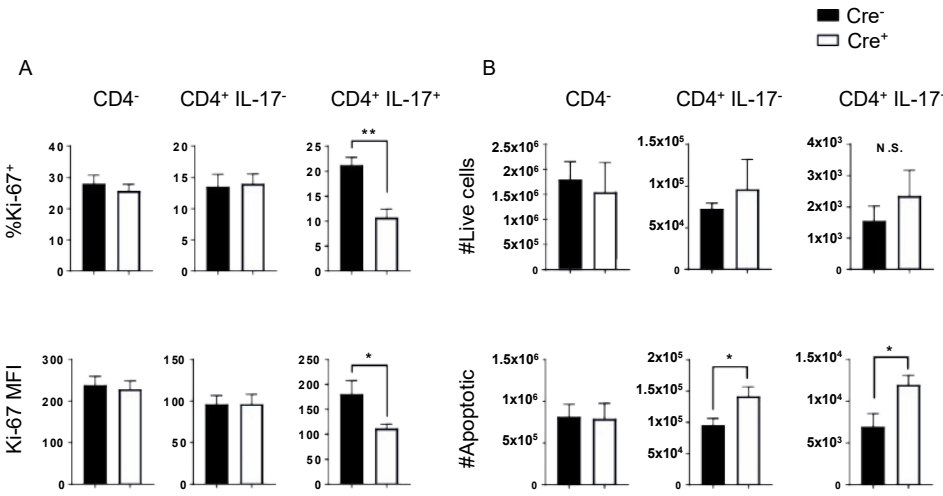


Figure 3. CD4^{+} T cell apoptosis is increased and Th17 cell proliferation is decreased in *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ mice compared with *CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$ *mice*. Flow cytometric analysis was conducted on SI-LP lymphocytes from naive *Cre*$^{-}$ and *Cre*$^{+}$ mice. Cells were first gated into CD4-, CD4+IL-17A-, and CD4+IL-17A+ populations for analysis. (A) Proliferation of each population was determined as the percentage expression and MFI of the proliferation marker Ki-67. (B) The number of live (Annexin V-negative viability dye-negative) and apoptotic (Annexin V-positive) cells within each population was calculated. Results are mean ± SEM in cells from 4 mice per group. *P < 0.05, **P < 0.01, by Student's t-test. See Figure 1 for definitions.

Partial reduction of acute AIA in Th17-deficient mice

To further examine the role of Th17 cells in arthritis, we first used mice with antigen-induced arthritis (AIA), an acute, non-immunogenic and non-autoimmune model [27, 28]. Development of AIA was previously shown to be dependent on the involvement of IL-17A [28]; however, the cellular source of IL-17A and the precise role of Th17 cells have not been elucidated. Comparison of AIA between *CD4-Cre*$^{+}$*Rorc*$^{flox/flox}$ and *CD4-Cre*$^{-}$*Rorc*$^{flox/flox}$ mice showed that the severity of arthritis was reduced in Cre^{+} mice compared with Cre^{-} mice (Supplementary Figure 4A). Control (PBS-injected) contralateral knees displayed minimal arthritis.

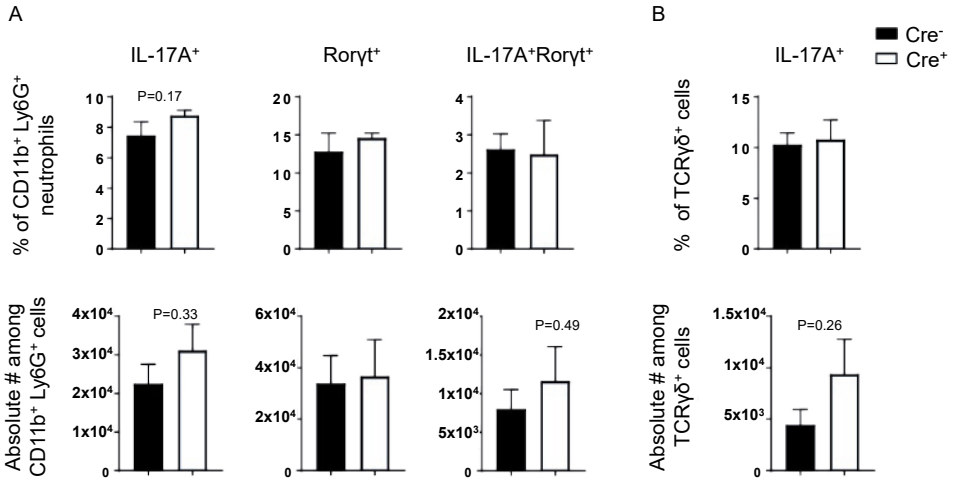


Figure 4. Production of IL-17 by SI-LP neutrophils and γδ T cells is not significantly altered in naive CD4-Cre$^+$ Rorc$^{flox/flox}$ or CD4-Cre$^-$ Rorc$^{flox/flox}$ mice. IL-17 production by intestinal neutrophils and γδ T cells from naive *Cre*$^-$ and *Cre*$^+$ mice was analyzed via flow cytometry. (A) Acquired cells were gated on neutrophils (CD11b$^+$ Ly6G$^+$) and the percent and absolute number of IL-17A$^+$, Rorγt$^+$, and IL-17A$^+$ Rorγt$^+$ neutrophils was determined. (B) SI-LP mononuclear cells were gated on TCRγδ$^+$ cells and the percentages and absolute numbers of IL-17$^+$ cells among TCRγδ$^+$ cells were analyzed. Results are mean ± SEM in cells from 4 mice per group. See Figure 1 for definitions.

Partial protection from arthritis development and progression in Cre$^+$ mice was confirmed by histology, which showed 40% reduction of synovial inflammation in Cre$^+$ mice compared with Cre$^-$ mice (Supplementary Figure 4B). There were no significant differences in cartilage and bone damage between the 2 groups, which is likely attributable to the acute nature of this model.

Analysis of the draining lymph nodes (DLN) showed significantly lower CD4$^+$ T cells frequency in the DLN of Cre$^+$ mice compared with Cre$^-$ mice (Supplementary Figure 4C). The frequency of Th17 cells in the DLNs was very low and similar in both groups. However, supernatant IL-17A concentration from DLN cells stimulated *ex vivo* with PMA and ionomycin were significantly decreased in Cre$^+$ mice compared with Cre$^-$ littermates (Supplementary Figure 4D).

We further investigated IL-17A expression by CD4$^-$ immune cells during the development of AIA. We found no significant difference in frequency or mean fluorescence intensity (MFI) of IL-17A within SI lamina propria CD11b$^+$ Gr1$^+$ neutrophils between Cre$^+$ and Cre$^-$ mice (Supplementary Figure 5A). These findings were confirmed in the MLN and DLN (data not shown). However, the numbers and frequencies of IL-17$^+$ CD8$^+$ T cells were lower, albeit non-significantly, in *CD4-Cre$^+$Rorc$^{flox/flox}$* mice with AIA compared with *CD4-Cre$^-$Rorc$^{flox/flox}$* mice with AIA (Supplementary Figures 5B-C). Although γδ T cells were not directly examined,, we observed that TCRβ$^-$ CD11b$^-$ CD11c$^-$ Gr1$^-$ population (likely representing γδ T cells) from *CD4-Cre$^+$Rorc$^{flox/flox}$* mice with AIA and *CD4-Cre$^-$Rorc$^{flox/flox}$* mice with AIA had similar levels of IL-17A expression (Supplementary Figure 5D).

Our data show that although Th17 cells enhance the development and progression of AIA, other important IL-17-producing cells may contribute to the remaining arthritis observed in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice (Supplementary Figure 4). These findings validate our hypothesis that Th17 cells play an important role in pathogenesis of arthritis and contribute to joint inflammation during acute AIA.

Suppression of CIA development in Th17-deficient mice

To further investigate the role of Th17 cells in chronic autoimmune arthritis, we induced CIA in *CD4-Cre^{-} Rorc$^{flox/flox}$* and *CD4-Cre^{+} Rorc$^{flox/flox}$* littermates. All of the mice developed arthritis in at least one paw. While the number of affected paws was similar between the groups, the number of affected knee joints was significantly lower in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice (mean ± SEM 1.25 ± 0.16) compared with *CD4-Cre^{-}Rorc$^{flox/flox}$* mice (mean ± SEM 1.78 ± 0.14). In SFB-specific qPCR analyses, we demonstrated that the mice harbored SFB (data not shown), a taxa known to induce the differentiation of Th17 cells and to exacerbate arthritis in K/BxN mice [9]. Nevertheless, studies have yet to demonstrate whether the involvement of these Th17 cells is required for arthritis, and the role of IL-17 in SFB-induced exacerbation of K/BxN arthritis has also been a subject of recent debate [45, 46]. Evaluation of CIA in our SFB-positive mice revealed that conditional Th17-deficient (Cre^{+}) mice had significantly lower arthritis scores compared with Cre^{-} littermate mice beginning 36 days after the primary immunization (Figure 5A). The severity of knee arthritis was also significantly lower in Cre^{+} mice compared with Cre^{-} mice at the study end point (Figure 5A).

Histopathologic examination of the ankle joints confirmed significantly less synovial inflammation, proteoglycan depletion, chondrocyte death, and cartilage erosion in Cre^{+} mice compared with Cre^{-} mice (Figures 5B-C). These data indicate that Th17 cells are required for the development and progression of chronic inflammatory arthritis in the CIA model.

To assess the effects of Th17 cell deficiency on the gut microbiota, we performed high-throughput 16S ribosomal RNA gene sequencing of fecal microbiota from *CD4-Cre^{+}Rorc$^{flox/flox}$* and *CD4-Cre^{-}Rorc$^{flox/flox}$* mice. The results showed that the relative abundance of the genus *Lactobacillus* was increased wheras the genera *Bacteroides*, *Enterococcus*, and *Candidatus arthromitus* (SFB) were reduced in *CD4-Cre^{+}Rorc$^{flox/flox}$* mice compared with *CD4-Cre^{-}Rorc$^{flox/flox}$* mice (Supplementary Figure 6 and Supplementary Table 3). These differences between the two groups were eliminated after correction for multiple testing.

A qPCR analysis of universal bacterial (Eubacteria) and SFB 16S genes showed that compared with naive mice, mice with CIA had a reduced universal bacterial 16S burden and an increased abundance of SFB (Supplementary Figure 7). Comparison of naive *Cre^{+}Rorc$^{flox/flox}$* mice and *CD4-Cre^{-}Rorc$^{flox/flox}$* mice revealed a non-signficant trend toward increased abundance of SFB in naive

Cre⁺Rorc$^{flox/flox}$ mice, which is consistent with previously reported findings in *CD4-Crre⁺STAT3$^{flox/flox}$* mice [47].In contrast, the levels of SFB were significantly decreased in Cre⁺ mice compared with Cre⁻ mice during the course of CIA (Supplementary Figure 7). Therefore, CD4-Cre-induced deletion of *Rorc* did not lead to a higher SFB burden in *Cre⁺Rorc$^{flox/flox}$* mice with arthritis.

Evidence that Th17 cell contributions to arthritis are microbiota-dependent.
Because the composition of the intestinal microbiota strongly influences induction of systemic and mucosal Th17 cells, we evaluated whether the requirement of Th17 cells for arthritis development was dependent on the microbiota present in the gut. We depleted the native (SFB⁺) microbiota of *CD4-Cre⁺ Rorc$^{flox/flox}$* mice and *CD4-Cre⁻ Rorc$^{flox/flox}$* mice with broad-spectrum antibiotics, and reconstituted the mice with SFB-free fecal microbiota obtained from Jackson mice [7]. After recolonization with SFB-free Jackson microbiota, CIA was induced and scores of arthritis severity were assessed. We observed no significant differences in arthritis severity for the duration of the experiment between Cre⁺ and Cre⁻ mice that had been reconstituted with Jackson microbiota (Figure 5D).
The composition of fecal microbiota observed in these mice was determined by 16S sequencing (Supplementary Figure 8). At the end point of the experiment, only two taxa in the microbiota differed significantly ($P<0.05$) between CD4-*Cre⁺ Rorc$^{flox/flox}$* mice and *CD4-Cre⁻ Rorc$^{flox/flox}$* mice reconstituted with Jackson microbiota (Supplementary Table 4). The significance of the difference was eliminated after correction for multiple testing. SFB were not detected by qPCR in the feces from Jackson microbiota-reconstituted mice at the end point of the experiment. These data suggest that the Th17 cell dependency of inflammatory arthritis relies on the microbiota-specific activation of mucosal Th17 cell immunity.

Disparate roles of Th17 cells and IL-17A in arthritis pathogenesis
Having established a contextual, microbiota-dependent role of Th17 cells during arthritis pathogenesis, we examined cytokine expression in the SI lamina propria and draining LNs of *CD4-Cre⁺ Rorc$^{flox/flox}$* and *CD4-Cre⁻ Rorc$^{flox/flox}$* mice with CIA. Consistent with our previous observations (see Figure 2), the frequency and MFI of IL-17A expression were decreased in SI lamina propria CD4⁺ T cells from Cre⁺ mice compared with Cre⁻ mice during CIA (Figure 6A).However, quantification of IL-17A from supernatants of SI lamina propria cells stimulated with PMA and ionomycin revealed that total SI lamina propria IL-17A production was not significantly affected in Th17 cell-deficient mice, and tended to be higher than that in Th17-sufficient mice ($P=0.63$) (Figure 6B). These findings suggest that non-Th17 cells, such as neutrophils and γδ T cells (see Figure 4), are the likely sources of IL-17 in *CD4-Cre⁺ Rorc$^{flox/flox}$* mice.

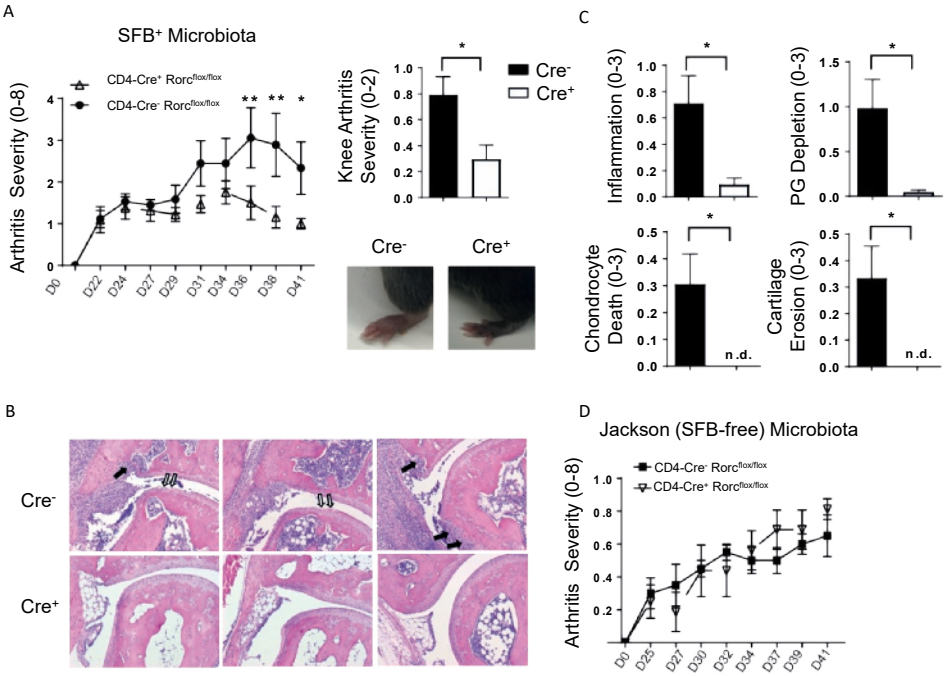


Figure 5. Th17 cells contribute to progression of collagen-induced arthritis (CIA) in a microbiota-dependent manner. CIA was induced in segemented filamentous bacteria-positive (SFB+) *CD4-Cre^{+} Rorc$^{flox/flox}$* and *CD4-Cre^{-} Rorc$^{flox/flox}$* mice. (A) Arthritis severity was scored in a blinded manner in Cre+ and Cre- mice 3 times per week, with a maximum score of 2 per limb and 8 per mouse (left panel). During necropsy, mouse knee joints (representative images shown in the lower right panels) were scored for arthritis severity on a scale of 0-2 (uper right panel). Results are the mean ± SEM scores of 8-9 mice per group. (B) Histologic images show features of arthritis in the ankle joints of a representative mouse from each group on day 41 of CIA (same experiment as shown in A). Cartilage erosion is indicated by open white arrows and bone erosion is indicated by solid black arrows. Original magnification x 100. (C) histological sections of the mouse ankle joints were scored on a scale of 0-3 for inflammation, proteoglycan (PG) depletion, chondrocyte death, and cartilage erosion. Results are the mean ± SEM scores of 16-18 joints per group. (D) *Cre^{+}* and *Cre^{-}* mice were depleted of native microflora and reconstituted with SFB-free microbiota from Jackson mice before the induction of CIA. Arthritis severity was scored in the same manner as described in A. Results are the mean ± SEM from a representative experiment in 4-5 mice per group.*P < 0.05, **P < 0.01, by Mann-Whitney U test (for 2 groups) or two-way repeated-measures analysis of variance with uncorected Fisher's least significant difference test (for groups over multiple time points). D = day; ND = not determined.

Similarly, the production of GM-CSF and IFNγ from SI lamina propria cells stimulated with PMA and ionomycin was not significantly different between the two genotypes (for GM-CSF; mean ± SEM 4.36±1.50 pg/mL in *Cre-* mice versus 3.92±1.26 pg/mL in *Cre+* mice; for IFNγ, 5.56±0.88 pg/mL, in *Cre-* mice versus 5.31±1.37 in *Cre+* mice). IL-17F and IL-22 were undetectable in culture supernatants of SI lamina propria cells (data not shown). Moreover, draining LN cells from *CD4-Cre$^-$ Rorc$^{flox/flox}$* and *CD4-Cre$^+$ Rorc$^{flox/flox}$* mice with CIA showed no significant difference in IL-17A production after stimulation with PMA and ionomycin (Figure 6C). Interestingly, the PMA and ionomycin-stimulated draining LNs from *Cre$^+$* mice had significantly higher concentrations of IFNγ compared with draining LNs from Cre$^-$ mice (Figure 6C). We observed no difference between the two genotypes in draining LNs cell production of GM-CSF (mean ± SEM 10.56±1.68 pg/mL in *Cre$^-$* mice versus 12.59±2.72 in *Cre$^+$* mice) or IL-17F (15.68±3.76 pg/mL in *Cre$^-$* mice versus 13.14±3.96 pg/mL in *Cre$^+$* mice). IL-22 was not detectable in the draining LNs. However, upon stimulation with CII to determine antigen-specific cytokine responses, IL-17A, IL-17F, and GM-CSF were all significantly lower in the drainig LNs of *Cre$^+$* mice compared with *Cre$^-$* mice (Figure 6D). Production of TNFα by CII-stimulated draining LNs was similar between the two groups, and IFNγ and IL-22 were not detectable following stimulation with CII (data not shown).

These data indicate that during CIA, there is no difference in total production of IL-17A, IL-17F, and GM-CSF by PMA and ionomycin-stimulated drainig LN cells between *Cre$^-$* mice and *Cre$^+$* mice. However, antigen-specific cytokine production is significantly reduced in *Cre$^+$* mice compared with *Cre$^-$* mice, a finding that suggests the potential involvement of Th17 cells. Thus, we may infer that the production of multiple cytokines including IL-17A, IL-17F, and GM-CSF; by Th17 cells drives arthritic joint inflammation in this murine model.

Discussion

The intestinal microbiome has emerged as a key determinant of health and disease. Culture-independent high-throughput sequencing techniques have revealed reduced diversity and significant overrepresentation of *Prevotella copri* in patients with new-onset, untreated RA [2, 3]. Another study found an association of treatment-naive RA with a cluster of metagenomic linkage groups related to *Clostridium asparagiforme*, *Gordonibacter pamelaeae*, *Eggerthella lenta* and *Lachnospiraceae* [4]. Furthermore, an increased abundance of *Collinsella*, *Eggerthella*, and *Faecalibacterium* was identified in patients with longstanding treated RA (mean disease duration of 81.6 months) [5]. However, the pathophysiological relevance of these microbiota alterations in new-onset or established RA is not fully understood.

A recent study demonstrated increased lamina propria Th17 cell differentiation

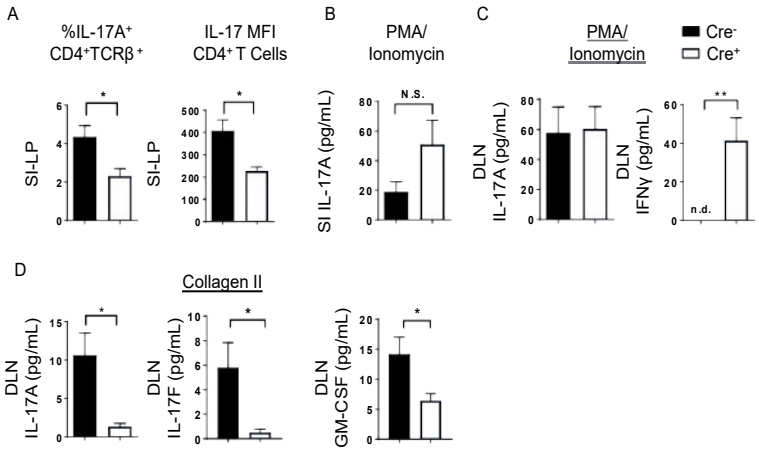


Figure 6. Protection from collagen-induced arthritis (CIA) in mice is dependent on the involvement of Th17 cells, but not the activity of IL-17A. CIA was induced in *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ and *CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$ mice, and cells from the SI-LP and draining LNs (DLNs) were analyzed for cytokine production. (A) Percentages of Th17 cells and the mean fluorescence intensity (MFI) of IL-17 expression were determined by flow cytometry in SI-LP CD4+ cells from *Cre*$^{+}$ and *Cre*$^{-}$ mice. Results are the mean ± SEM in cells of 4 mice per group. (B) Production of IL-17A was determined in SI-LP cells cultured with PMA and ionomycin. Results are the mean ± SEM 2-3 replicate experiments in cells from 4-6 mice per group. (C) Production of IL-17A and IFNγ was determined in DLN lymphocytes ($2x10^5$ cells/well) that were stimulated with PMA and ionomycin for 6 hours. Results are the mean ± SEM of 2-3 replicate experiments in cells from 4-8 mice per group. (D) Production of IL-17A, IL-17F, and GM-CSF production was determined in supernatants of DNLs lymphocytes ($2x10^5$ cells/well) that were stimulated with type II collagen for 2 days. Results are the mean ± SEM of 1-2 replicate experiments in cells from 5-8 mice per group. All cytokines were measured by Luminex cytokine array. *P < 0.05; **P<0.01, by Mann-Whitney U test. N.S. = not significant

in SKG mice humanized with fecal microbiota from patients with new-onset RA compared with mice harbouring fecal microbiota from healthy controls [3]. This coincided with more severe arthritis in the SKG mice when mice were co-exposed to the fungal component zymosan [3]. However, a requirement for the involvement of microbiota-induced Th17 cells was not demonstrated. While several studies have demonstrated that experimental arthritis can be exacerbated by SFB [9, 32, 45, 46], it is unclear whether these arthritogenic effects are due to Th17 cell induction or due to T follicular helper cell induction and autoantibody production [45]. Microbiome perturbations occur during CIA and composition differs between both naive and arthritic CIA-susceptible and -resistant mouse strains [48-50]. Tuftsin phosphorylcholine, a natural glycoprotein with tolerogenic potential, attenuates the development of CIA and prevents dysbiosis in mice [48, 51]. Studies on microbiota-dependent differentiation of Th17 in the K/BxN model of arthritis have demonstrated that Th17 cell induction in SFB-colonized mice occurs around weaning and precedes the onset of arthritis [9, 46]. Because age-dependent increases in SI lamina propria Th17 cells are also observed in

in naive mice, a side-by-side comparison between age-matched prearthritic mice and naive control mice, as was performed in the present study, is crucial to demonstrate that the increase in Th17 cells is characteristic of preclinical arthritis in mice.

Our findings demonstrate initiation of mucosal Th1 and Th17 cell responses and marked production of TNFα, GM-CSF, and IL-22 by mucosal CD4 T cells just before clinical onset of arthritis. Furthermore, increased production of GM-CSF and IL-22 by SI lamina propria Th17 cells during preclinical arthritis is an additional novel finding. These cytokines are implicated in the pathogenesis of RA and may have IL-17–independent pro-inflammatory and osteoclastogenic functions [13, 20-22]. Therefore, our observations suggest that activation of intestinal mucosal T cells during the immune-priming phase of RA may contribute to early, preclinical processes.

Further studies using Th17-deficient mice showed that Th17 cells are required for the progression of arthritis and affect several features of structural joint pathology in two murine arthritis models, mBSA-induced arthritis (AIA) and CIA. AIA is an acute, nonimmunogenic and nonautoimmune model of arthritis, whereas CIA is chronic, immunogenic, and autoimmune. Chronic acute autoimmunity models frequently demonstrate differential immunogenicity, cellular involvement, and mechanisms of pathogenesis. Therefore, these two models provide complementary insights into the role of Th17 cells in arthritis. The known role of other cellular sources of IL-17 in arthritis [52] emphasizes the value of investigating the specific, differential contribution of Th17 cells as a main source of IL-17 and other proinflammatory mediators.

In our comparison of the effects of CD4-induced *Rorc* deletion on the acute AIA and CIA models, we found that the AIA model is less dependent on Th17 cells. This aligns with previous observations of the role of the cytokine IL-17A in these two models [28, 35, 53, 54]. This may be attributed to the B cell and antibody dependence of CIA as compated to acute AIA, because Th17 cells also assist B cells and promote antibody production. Conversely, AIA could reflect a predominant role of other IL-17-producing cells, such as $\gamma\delta$ T cells. Examining $\gamma\delta$ T cell-specific *Rorc* deletion during CIA and AIA is an avenue for future study.

Since total IL-17 production upon PMA and ionomycin stimulation was not affected in arthritis-resistant Th17-deficient mice, it is likely that the role of Th17 cells in arthritis expands beyond that of the cytokine IL-17. Moreover, the cytokine profile of collagen-stimulated cells from joint-draining LNs indicated that antigen-experienced T cells produced IL-17A, IL-17F, and GM-CSF, whereas the levels of these were significantly reduced in Th17 cell-deficient mice. GM-CSF activates the monocyte/macrophage system as well as neutrophils and promotes inflammation [20]. Inhibition of GM-CSF or its receptor led to a reduction in the RA disease activity score and C-reactive protein levels in clinical trials [20, 55, 56]. GM-CSF and IL-17A act synergistically to induce matrix metalloproteinases,

RANKL and IL-23 in synovium, and simultaneous expression of IL-17A and GM-CSF leads to complete destruction of joint structure in mice [57]. Accordingly, simultaneous blockade of IL-17A and GM-CSF is more effective than blocking either cytokine alone [57]. Therefore, we attribute the reduced arthritis severity in *Cre*$^{+}$ mice to the simultaneous reduction of IL-17A, IL-17F and GM-CSF production by antigen-specific Th17 cells.

Th17 production of multiple proinflammatory cytokines with synergistic effects during arthritis suggests that modulation of Th17 cell development may be more effective than IL-17 blockade. This may extend to other rheumatic inflammatory arthritis such as psoriatic arthritis in which Th17 cells play a pathogenic role [58]. In addition, direct effects of Th17 cells on B cell activation and isotype class switching as well as facilitation of antibody production have been reported, and may represent other important IL-17-independent roles for Th17 cells [59, 60]. While T cell IL-22 expression was increased in immunized mice during preclinical arthritis (Figure 1B-D), IL-22 was not detectable in culture supernatants derived from the SI lamina propria or draining LNs of mice with fulminant CIA. Therefore, the role of IL-22-producing mucosal T cells in arthritis remains unclear and may be restricted to the preclinical phase.

Differentiation of the Th17 lineage is mediated by both RORα and RORγt transcription factors [61]. Intact RORα expression in CD4-expressing cells may explain why the Th17 cell deficiency in *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ mice is incomplete. Studies of RORα/RORγt double-deficient mice with fully impaired Th17 development [61] would help clarify whether arthritis is fully inhibited in the complete absence of Th17 cells.

Th17 cells and IL-17 production are strongly induced by intestinal microbiota [8, 9, 23]. Therefore, the microbiota composition strongly influences the dominant immune processes underlying disease in certain patients. Th17 frequencies are elevated in RA, particularly in patients with poor responses to TNF inhibitors [13, 62]. Our data show that in the absence of specific murine Th17 cell-inducing microbiota, namely SFB, the Th17 dependence of arthritis is mitigated. Therefore, Th17 cells contribute to the pathogenesis of arthritis only in the context of specific microbiota. This suggests that the composition of the microbiota in each patient may be a major factor affecting the involvement of the Th17/IL-17 pathway in mucosal immunity and RA. Thus, the microbiome profile of a patient may be a valuable biomarker for predicting the efficacy of Th17 cell- or IL-17-targeted therapies. We therefore speculate that microbiome-guided stratification of patients with inflammatory arthritis may improve therapeutic efficacy.

References

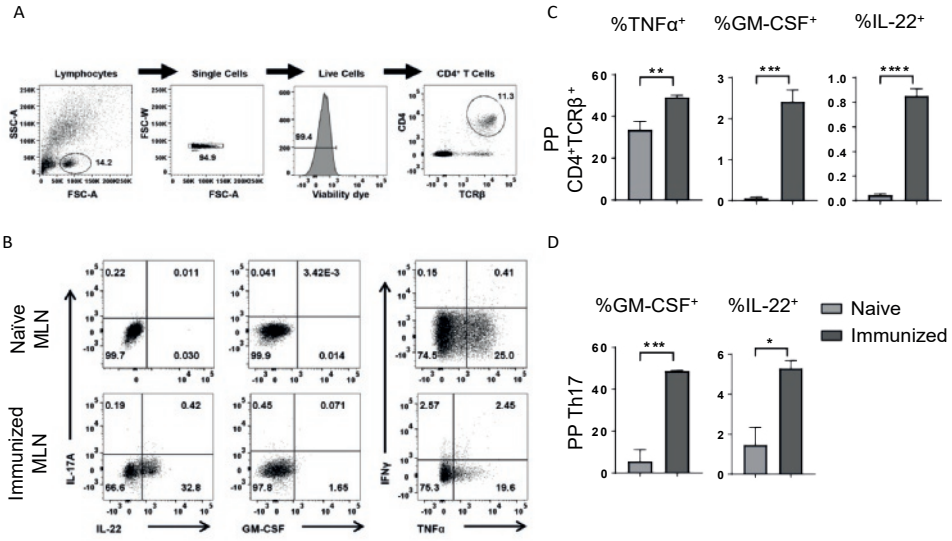
1. Rosenbaum JT, Asquith MJ. The Microbiome: a Revolution in Treatment for Rheumatic Diseases? Curr Rheumatol Rep. 2016;18(10):62.
2. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife. 2013;2:e01202.
3. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T cells in the intestine. Arthritis & rheumatology. 2016.
4. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nature medicine. 2015;21(8):895-905.
5. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome Med. 2016;8(1):43.
6. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75-84.
7. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485-98.
8. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009;31(4):677-89.
9. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
10. Rogier R, Ederveen THA, Boekhorst J, Wopereis H, Scher JU, Manasson J, et al. Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis. Microbiome. 2017;5(1):63.
11. Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, et al. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature. 2003;426(6965):454-60.
12. Kugyelka R, Kohl Z, Olasz K, Mikecz K, Rauch TA, Glant TT, et al. Enigma of IL-17 and Th17 Cells in Rheumatoid Arthritis and in Autoimmune Animal Models of Arthritis. Mediators of inflammation. 2016;2016:6145810.
13. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. Nature reviews Rheumatology. 2015;11(7):415-29.
14. Lee H, Jin BE, Jang E, Lee AR, Han DS, Kim HY, et al. Gut-residing Microbes Alter the Host Susceptibility to Autoantibody-mediated Arthritis. Immune Netw. 2014;14(1):38-44.
15. Cho WS, Jang E, Kim HY, Youn J. Interleukin 17-expressing Innate Synovial Cells Drive K/Bxn Serum-induced Arthritis. Immune Netw. 2016;16(6):366-72.
16. Genovese MC, Greenwald M, Cho CS, Berman A, Jin L, Cameron GS, et al. A phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. Arthritis & rheumatology. 2014;66(7):1693-704.
17. Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Aelion JA, et al. One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study. The Journal of rheumatology. 2014;41(3):414-21.
18. Burmester GR, Durez P, Shestakova G, Genovese MC, Schulze-Koops H, Li Y, et al. Association of HLA-DRB1 alleles with clinical responses to the anti-interleukin-17A monoclonal antibody secukinumab in active rheumatoid arthritis. Rheumatology. 2016;55(1):49-55.

19. Kim KW, Kim HR, Park JY, Park JS, Oh HJ, Woo YJ, et al. Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts. Arthritis and rheumatism. 2012;64(4):1015-23.
20. Avci AB, Feist E, Burmester GR. Targeting GM-CSF in rheumatoid arthritis. Clin Exp Rheumatol. 2016;34(4 Suppl 98):39-44.
21. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. Nature reviews Rheumatology. 2016;12(1):37-48.
22. van den Berg WB, McInnes IB. Th17 cells and IL-17 a--focus on immunopathogenesis and immunotherapeutics. Semin Arthritis Rheum. 2013;43(2):158-70.
23. Shaw MH, Kamada N, Kim YG, Nunez G. Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. The Journal of experimental medicine. 2012;209(2):251-8.
24. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity. 2001;15(5):763-74.
25. Takeda Y, Kang HS, Freudenberg J, DeGraff LM, Jothi R, Jetten AM. Retinoic acid-related orphan receptor gamma (RORgamma): a novel participant in the diurnal regulation of hepatic gluconeogenesis and insulin sensitivity. PLoS Genet. 2014;10(5):e1004331.
26. Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N, et al. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. Infect Immun. 2008;76(3):907-15.
27. Lawlor KE, Campbell IK, O'Donnell K, Wu L, Wicks IP. Molecular and cellular mediators of interleukin-1-dependent acute inflammatory arthritis. Arthritis and rheumatism. 2001;44(2):442-50.
28. Egan PJ, van Nieuwenhuijze A, Campbell IK, Wicks IP. Promotion of the local differentiation of murine Th17 cells by synovial macrophages during acute inflammatory arthritis. Arthritis and rheumatism. 2008;58(12):3720-9.
29. Harre U, Lang SC, Pfeifle R, Rombouts Y, Fruhbeisser S, Amara K, et al. Glycosylation of immunoglobulin G determines osteoclast differentiation and bone loss. Nat Commun. 2015;6:6651.
30. Campbell IK, Hamilton JA, Wicks IP. Collagen-induced arthritis in C57BL/6 (H-2b) mice: new insights into an important disease model of rheumatoid arthritis. Eur J Immunol. 2000;30(6):1568-75.
31. Abdollahi-Roodsaz S, Joosten LA, Roelofs MF, Radstake TR, Matera G, Popa C, et al. Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. Arthritis and rheumatism. 2007;56(9):2957-67.
32. Chappert P, Bouladoux N, Naik S, Schwartz RH. Specific gut commensal flora locally alters T cell tuning to endogenous ligands. Immunity. 2013;38(6):1198-210.
33. Rosser EC, Oleinika K, Tonon S, Doyle R, Bosma A, Carter NA, et al. Regulatory B cells are induced by gut microbiota-driven interleukin-1beta and interleukin-6 production. Nature medicine. 2014;20(11):1334-9.
34. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. Journal of immunology. 2003;171(11):6173-7.
35. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis and rheumatism. 2004;50(2):650-9.
36. Koenders MI, Lubberts E, van de Loo FA, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, et al. Interleukin-17 acts independently of TNF-alpha under arthritic conditions. Journal of immunology. 2006;176(10):6262-9.
37. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med. 2009;361(9):888-98.
38. Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, Leipe J, et al. Mast cells express IL-17A in rheumatoid arthritis synovium. Journal of immunology. 2010;184(7):3336-40.

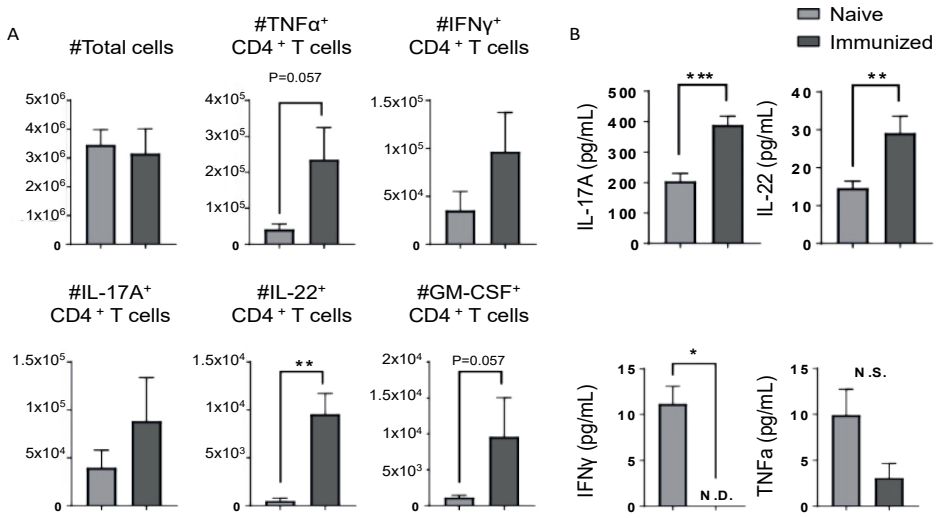
39. van Baarsen LG, Lebre MC, van der Coelen D, Aarrass S, Tang MW, Ramwadhdoebe TH, et al. Heterogeneous expression pattern of interleukin 17A (IL-17A), IL-17F and their receptors in synovium of rheumatoid arthritis, psoriatic arthritis and osteoarthritis: possible explanation for nonresponse to anti-IL-17 therapy? Arthritis research & therapy. 2014;16(4):426.
40. Kyburz D, Corr M. The KRN mouse model of inflammatory arthritis. Springer Semin Immunopathol. 2003;25(1):79-90.
41. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity. 1999;10(4):451-61.
42. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126(6):1121-33.
43. Wu H, Xie MM, Liu H, Dent AL. Stat3 Is Important for Follicular Regulatory T Cell Differentiation. PloS one. 2016;11(5):e0155040.
44. Ye J, Wang Y, Liu X, Li L, Opejin A, Hsueh EC, et al. TLR7 Signaling Regulates Th17 Cells and Autoimmunity: Novel Potential for Autoimmune Therapy. Journal of immunology. 2017;199(3):941-54.
45. Block KE, Zheng Z, Dent AL, Kee BL, Huang H. Gut Microbiota Regulates K/BxN Autoimmune Arthritis through Follicular Helper T but Not Th17 Cells. Journal of immunology. 2016.
46. Teng F, Klinger CN, Felix KM, Bradley CP, Wu E, Tran NL, et al. Gut Microbiota Drive Autoimmune Arthritis by Promoting Differentiation and Migration of Peyer's Patch T Follicular Helper Cells. Immunity. 2016;44(4):875-88.
47. Kumar P, Monin L, Castillo P, Elsegeiny W, Horne W, Eddens T, et al. Intestinal Interleukin-17 Receptor Signaling Mediates Reciprocal Control of the Gut Microbiota and Autoimmune Inflammation. Immunity. 2016;44(3):659-71.
48. Ben-Amram H, Bashi T, Werbner N, Neuman H, Fridkin M, Blank M, et al. Tuftsin-Phosphorylcholine Maintains Normal Gut Microbiota in Collagen Induced Arthritic Mice. Front Microbiol. 2017;8:1222.
49. Liu X, Zeng B, Zhang J, Li W, Mou F, Wang H, et al. Role of the Gut Microbiome in Modulating Arthritis Progression in Mice. Sci Rep. 2016;6:30594.
50. Gomez A, Luckey D, Yeoman CJ, Marietta EV, Berg Miller ME, Murray JA, et al. Loss of sex and age driven differences in the gut microbiome characterize arthritis-susceptible 0401 mice but not arthritis-resistant 0402 mice. PloS one. 2012;7(4):e36095.
51. Bashi T, Shovman O, Fridkin M, Volkov A, Barshack I, Blank M, et al. Novel therapeutic compound tuftsin-phosphorylcholine attenuates collagen-induced arthritis. Clin Exp Immunol. 2016;184(1):19-28.
52. Katayama M, Ohmura K, Yukawa N, Terao C, Hashimoto M, Yoshifuji H, et al. Neutrophils are essential as a source of IL-17 in the effector phase of arthritis. PloS one. 2013;8(5):e62231.
53. Pollinger B, Junt T, Metzler B, Walker UA, Tyndall A, Allard C, et al. Th17 cells, not IL-17+ gammadelta T cells, drive arthritic bone destruction in mice and humans. Journal of immunology. 2011;186(4):2602-12.
54. Sarkar S, Justa S, Brucks M, Endres J, Fox DA, Zhou X, et al. Interleukin (IL)-17A, F and AF in inflammation: a study in collagen-induced arthritis and rheumatoid arthritis. Clin Exp Immunol. 2014;177(3):652-61.
55. Burmester GR, McInnes IB, Kremer J, Miranda P, Korkosz M, Vencovsky J, et al. A randomised phase IIb study of mavrilimumab, a novel GM-CSF receptor alpha monoclonal antibody, in the treatment of rheumatoid arthritis. Annals of the rheumatic diseases. 2017;76(6):1020-30.
56. Huizinga TW, Batalov A, Stoilov R, Lloyd E, Wagner T, Saurigny D, et al. Phase 1b randomized, double-blind study of namilumab, an anti-granulocyte macrophage colony-stimulating factor monoclonal antibody, in mild-to-moderate rheumatoid arthritis. Arthritis research & therapy. 2017;19(1):53.

57. van Nieuwenhuijze AE, van de Loo FA, Walgreen B, Bennink M, Helsen M, van den Bersselaar L, et al. Complementary action of granulocyte macrophage colony-stimulating factor and interleukin-17A induces interleukin-23, receptor activator of nuclear factor-kappaB ligand, and matrix metalloproteinases and drives bone and cartilage pathology in experimental arthritis: rationale for combination therapy in rheumatoid arthritis. Arthritis research & therapy. 2015;17:163.
58. Benham H, Norris P, Goodall J, Wechalekar MD, FitzGerald O, Szentpetery A, et al. Th17 and Th22 cells in psoriatic arthritis and psoriasis. Arthritis research & therapy. 2013;15(5):R136.
59. Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK, et al. Proinflammatory T helper type 17 cells are effective B-cell helpers. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(32):14292-7.
60. Shibui A, Shimura E, Nambu A, Yamaguchi S, Leonard WJ, Okumura K, et al. Th17 cell-derived IL-17 is dispensable for B cell antibody production. Cytokine. 2012;59(1):108-14.
61. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity. 2008;28(1):29-39.
62. Chen DY, Chen YM, Chen HH, Hsieh CW, Lin CC, Lan JL. Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF-alpha therapy. Arthritis research & therapy. 2011;13(4):R126.

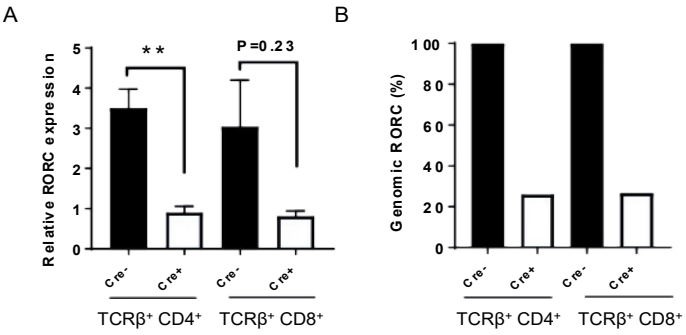
Supplementary material



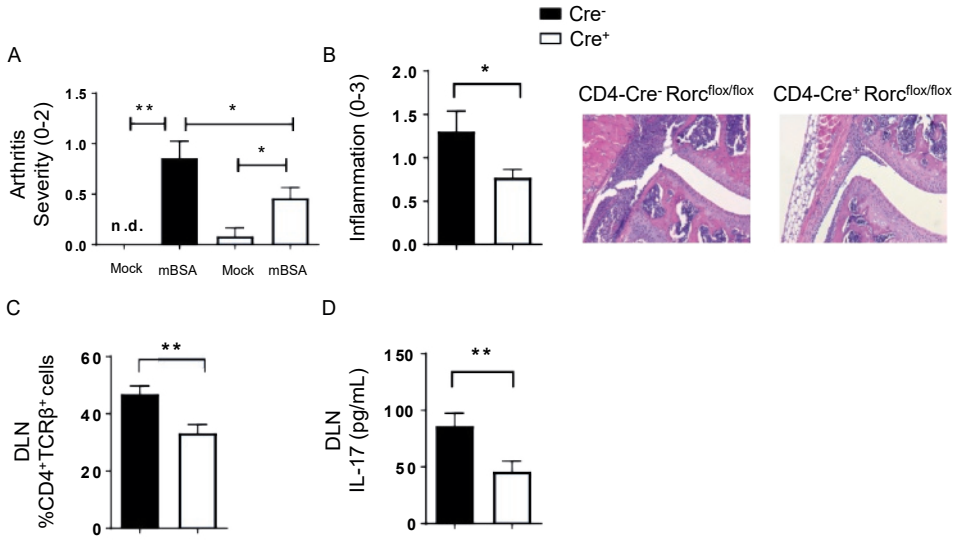
Supplementary Figure 1. Immunization with type II collagen induces CD4⁺ T cell responses in the gut. SI-LP, MLN, and PP lymphocytes from naive and immunized WT mice were isolated, re-stimulated with PMA and ionomycin, and stained for intracellular flow cytometry as described. (A) Flow cytometry gating strategy for identifying CD4⁺ T cells. (B) Representative flow cytometry plots of IFNγ, TNFα, GM-CSF, IL-17, and IL-22 expression by CD4⁺ T cells in the MLNs of naive and immunized mice. (C) Bar graphs showing percent expression of TNFα, GM-CSF, and IL-22 production by CD4⁺ T cells from the PP of naive and immunized mice. (D) Percent expression of GM-CSF and IL-22 production by Th17 cells in the PP of naive and immunized mice. Statistical analysis was performed using a Student's T test: *P < 0.05, **P < 0.01, ***P < 0.001, ****P<0.0001.



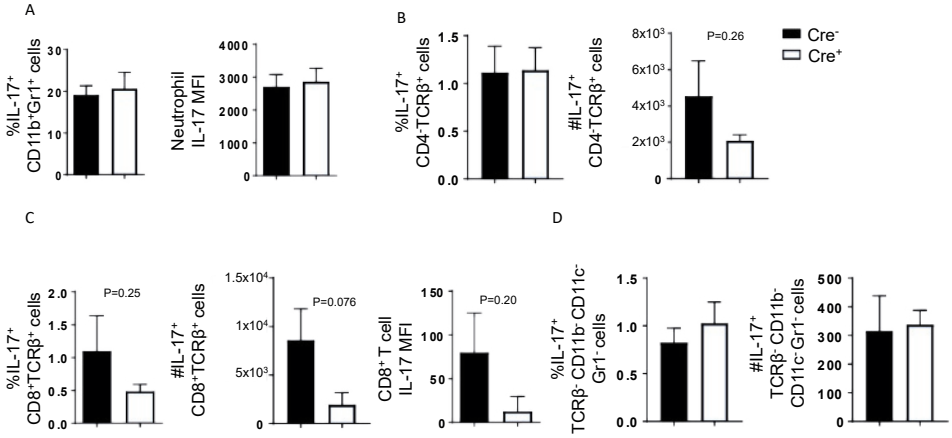
Supplementary Figure 2. Intestinal immune activation occurs during the preclinical phase of arthritis. SI-LP mononuclear cells were isolated from naive and single-immunized (non-arthritic) WT mice. The number of total SI-LP cells was counted via hemocytometer. (A) Absolute numbers of TNFα$^+$, IFNγ$^+$, IL-17A$^+$, IL-22$^+$, and GM-CSF$^+$ CD4$^+$ T cells were calculated using values obtained from flow cytometric analysis. (B) Concentration of IL-17A, IL-22, TNFα, and IFNγ was measured in the supernatants of SI-LP lymphocytes (10^5 cells/well) stimulated with PMA and ionomycin for 5 hours *P < 0.05, **P < 0.01, ***P < 0.001, N.S.=not significant



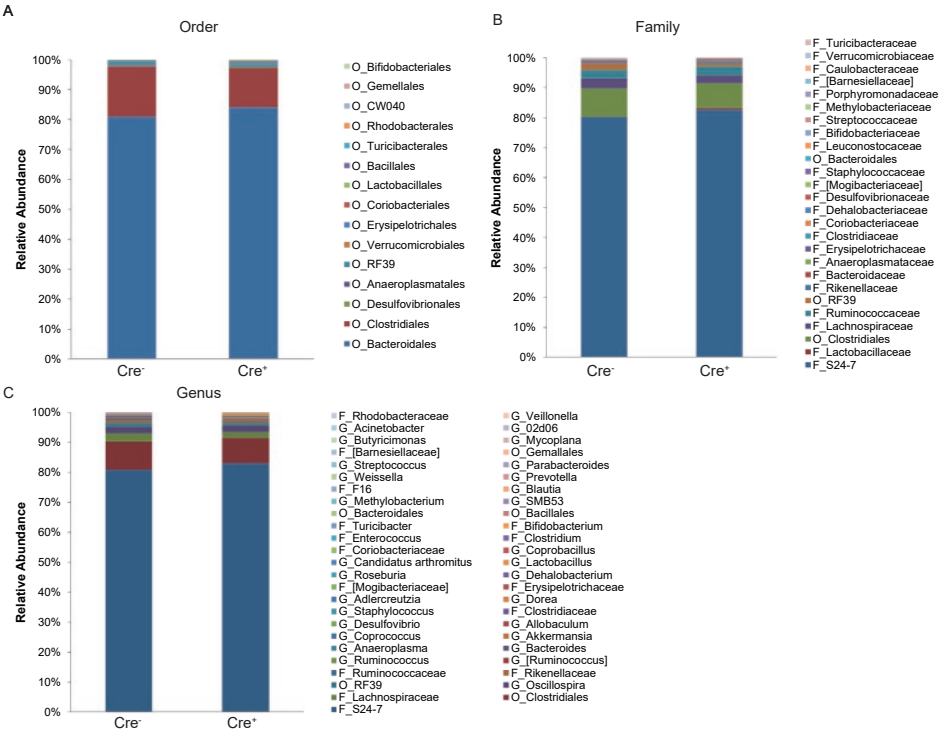
Supplementary Figure 3. Efficient deletion of *Rorc* in CD4-T cells. Genomic DNA was extracted from sorted CD4$^+$ and CD8$^+$ splenocytes from naive *CD4-Cre$^-$ Rorc$^{flox/flox}$* and *CD4-Cre$^+$ Rorc$^{flox/flox}$* mice. The *Rorc* gene was amplified via qPCR and normalized to *periostin* genomic DNA. (A) Relative amount of genomic *Rorc* normalized to periostin and (B) deletion efficiency of the Rorc gene are shown. **P < 0.01



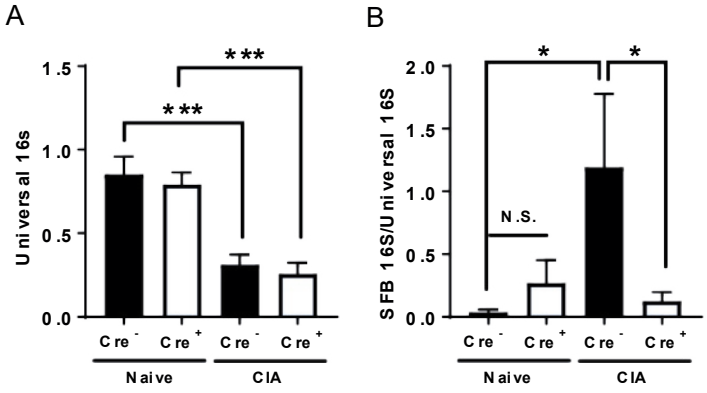
Supplementary Figure 4. Conditional Th17 deficiency results in protection from antigen-induced arthritis (AIA). AIA was induced *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ and *CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$ mice for 7 days. (A) Arthritis severity in each knee was scored during necropsy on a scale of 0-2 by investigators blinded for the genotype and the type of injection. Data represent 14 mBSA-injected and 4 mock (saline-injected) control joints. (B) Knee joint sections were stained with H&E and analyzed for histological indicators of arthritis. Mean + SEM of inflammation scores and representative histology images at 50x magnification are shown. Data are representative of 14 mice per group. (C) Flow cytometric analysis representing mean + SEM of percent CD4^{+} T cells (CD4^{+}TCRβ^{+}) in joint-draining lymph nodes (DLN) within the total live cell gate. (D) Mean concentration of IL-17A (pg/mL) from the supernatants of DLN cells (2x10^{5}/well) stimulated with PMA and ionomycin for 6 hours. Statistical analysis was performed via Mann-Whitney U test (to compare 2 groups) or non-parametric ANOVA with uncorrected Dunn's test (to compare 3 or more groups): *P < 0.05, **P < 0.01.



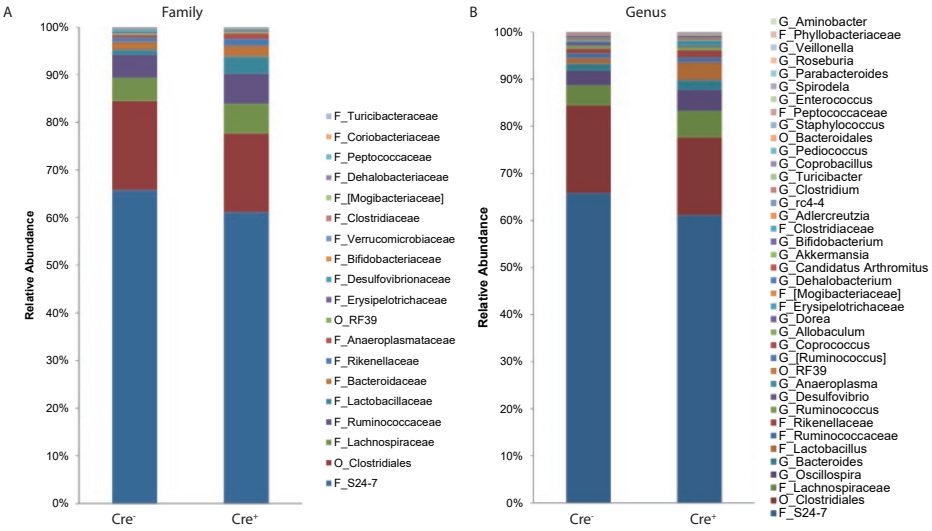
Supplementary Figure 5. IL-17 production from non-Th17 sources is unaltered in Th17-deficient mice during arthritis. AIA was induced in *CD4-Cre*$^{+}$ *Rorc*$^{flox/flox}$ and *CD4-Cre*$^{-}$ *Rorc*$^{flox/flox}$ mice as described and SI-LP lymphocytes were isolated and stained for flow cytometry. (A) Neutrophil IL-17 production was examined by gating on CD11b^{high} Gr1^{+} cells and analyzing percent expression and MFI of IL-17. (B) IL-17 production by total CD4^{-} T cells was analyzed by gating on TCRβ^{+}, CD4^{-} live cells and analyzing percent and absolute number of IL-17^{+} cells within the parent population. (C) IL-17 production by CD8^{+} T cell was examined by gating on CD8^{+} TCRβ^{+} cells and analyzing absolute number, percent expression, and MFI of IL-17^{+} cells within the parent population. (D) Percent and absolute number of IL-17^{+} cells among cells negative for the lineage markers TCRβ, CD11b, CD11c, and Gr1; which likely represent γδ T cells. Data represent 5 mice per group. Statistical analysis was performed via Mann-Whitney U test.



Supplementary Figure 6. Microbiota composition from *CD4-Cre⁻Rorc$^{flox/flox}$* and *CD4-Cre⁺Rorc$^{flox/flox}$* mice with CIA. Composition of the intestinal microbiota of *CD4-Cre⁻Rorc$^{flox/flox}$* and *CD4-Cre⁺Rorc$^{flox/flox}$* mice was determined via 16S rRNA gene sequencing of fecal microbial DNA. Relative abundance of bacterial taxa at the (A) order, (B) family, and (C) genus levels are shown. Feces were collected at day 41 of CIA, which was the endpoint of the experiment shown in Figure 4A. O=Order, F=Family, G=Genus



Supplementary Figure 7. Total bacteria and SFB in feces from naive and arthritic *CD4-Cre⁻Rorc$^{flox/flox}$* and *CD4-Cre⁺Rorc$^{flox/flox}$* mice. (A) Fecal DNA from *CD4-Cre⁻Rorc$^{flox/flox}$* and *CD4-Cre⁺Rorc$^{flox/flox}$* mice with and without CIA was amplified via qPCR with universal and SFB-specific 16S primer sets. To determine the total bacterial load, 16S amplification was normalized to the DNA concentration of the sample. (B) The relative abundance of SFB was determined via a ratio of SFB-specific 16S amplification to total bacterial 16S. *P < 0.05, ***P < 0.001, N.S.=not significant



Supplementary Figure 8. Microbiota composition from Jackson microbiota-reconstituted *CD4-Cre^{-}Rorc$^{flox/flox}$* and *CD4-Cre^{+}Rorc$^{flox/flox}$* mice with CIA. Composition of the intestinal microbiota was determined via 16S rRNA gene sequencing of fecal microbial DNA from *CD4-Cre^{-}Rorc$^{flox/flox}$* and *CD4-Cre^{+}Rorc$^{flox/flox}$* mice at day 41 of CIA, which was the endpoint of the experiment shown in Figure 4D. Relative abundance of bacterial taxa at the (A) family and (B) genus levels are shown. O=Order, F=Family, G=Genus.

Supplementary Table 1. Flow cytometry antibodies and dyes.

Target / Dye	Clone	Fluorochrome	Manufacturer
annexin V	N/A	PE	eBioscience
CD11b	M1/70	Brilliant Violet 650	BioLegend
CD4	RM4-5	PE/Cy7	eBioscience
CD4	GK1.5	APC	eBioscience
CD8α	53-6.7	PerCP/Cy5.5	eBioscience
CD8α	53-6.7	Brilliant Violet 785	BioLegend
Fixable viability dye	N/A	eflour 506	eBioscience
GM-CSF	MP1-22E9	FITC	BioLegend
Gr1	RB6-8C5	AlexaFlour700	eBioscience
IFNγ	XMG1.2	APC	eBioscience
IL-17	eBio17B7	PE	eBioscience
IL-17A	TC11-18H10.1	Pacific Blue	BioLegend
IL-22	1H8PWSR	PerCP/Cy5.5	eBioscience
Ki-67	B56	AlexaFlour700	BD
Ly6G	1A8	Brilliant Violet 785	BioLegend
RORγt	AFKJS-9	PE	eBioscience
TCRβ	H57-597	FITC	eBioscience
TCRβ	H57-597	Brilliant Violet 421	BioLegend
TCRγδ	GL-3	APC	eBioscience
TNFα	MP6-XT22	PE/Dazzle 594	BioLegend

Supplementary Table 2. Primer pairs for amplification of genomic DNA.

Gene	Taxonomic specificity	Forward primer	Reverse primer
16s	SFB	5'-GACGCTGAGGCATGAGAGCAT-3'	5'-GACGGCACGGATTGTTATTCA-3'
16s	Eukaryote	340F	514R
16s	Eukaryote/ Archea	515F	806R
Periostin	Mouse	5’-CATCCTAAATACCCTCCAGTGC-3’	5’-GGACTTCATCAATCAGGTGGA-3’
Rorc	Mouse	5'-TTCCTTCCTTCTTCTTGAGCAGTC-3'	5'-CAGAAGAAAAGTATATGTGGCTTGTTG-3'

Supplementary Table 3. Differentially abundant operational taxonomic units in fecal microbiota of *CD4-Cre^{-}Rorc$^{flox/flox}$* versus *CD4-Cre^{+}Rorc$^{flox/flox}$* mice with CIA. Relative abundance (percent), expressed as mean and SEM, of microbial taxa that are differentially present in the feces of *CD4-Cre^{-}Rorc$^{flox/flox}$* and *CD4-Cre^{+}Rorc$^{flox/flox}$* mice with CIA. Feces were collected at day 41 of CIA, which was the endpoint of the experiment shown in Figure 4A. P values were calculated via Student's T test and a P value of less than 0.05 was considered significant. These values were not significant when correcting for a false discovery rate (α=0.10) with the Benjamini-Hochberg procedure. O=Order, F=Family, G=Genus

Taxanomic ID	Cre^{-} mean	Cre^{-} SEM	Cre^{+} mean	Cre^{+} SEM	P value
F_Lactobacillaceae	0.0235	0.0108	0.4817	0.2989	0.0093
G_Lactobacillus	0.0235	0.0108	0.4817	0.2989	0.0093
F_Bacteroidaceae	0.3152	0.1665	0.0010	0.0005	0.0174
G_Bacteroides	0.3152	0.1665	0.0010	0.0005	0.0174
G_Enterococcus	0.0076	0.0044	0.0000	0.0000	0.0337
O_RF39	1.3580	0.2029	0.8386	0.1341	0.0360
P_Verrucomicrobia	0.2111	0.2627	0.0867	0.0929	0.0364
O_Lactobacillales	0.0323	0.0105	0.4825	0.2996	0.0401
G_Candidatus arthromitus	0.0178	0.0063	0.0029	0.0025	0.0438

Supplementary Table 4. Differentially abundant operational taxonomic units in the microbiota of Jackson-microbiota reconstituted *CD4-Cre^{-}Rorc$^{flox/flox}$* versus *CD4-Cre^{+}Rorc$^{flox/flox}$* mice with CIA. Relative abundance (percent), expressed as mean and SEM, of microbial taxa that are differentially present in the feces of *CD4-Cre^{-}Rorc$^{flox/flox}$* and *CD4-Cre^{+}Rorc$^{flox/flox}$* mice with CIA and Jackson microbiota. Feces were collected at day 41 of CIA, which was the endpoint of the experiment. P values were calculated via Student's T test and a P value of less than 0.05 was considered significant. These values were not significant when correcting for a false discovery rate (α=0.10) with the Benjamini-Hochberg procedure. O=Order, F=Family, G=Genus

Taxanomic ID	Cre^{-} mean	Cre^{-} SEM	Cre^{+} mean	Cre^{+} SEM	P value
F_Rikenellaceae	0.9457	0.1214	1.4609	0.0859	0.0250
G_Parabacteroides	0	0	0.001562	0.0007864	0.0346

Chapter 6

Supplementation of diet with non-digestible oligosaccharides alters the intestinal microbiota, but not arthritis development, in IL-1 receptor antagonist deficient mice

Chapter 6

Supplementation of diet with non-digestible oligosaccharides alters the intestinal microbiota, but not arthritis development, in IL-1 receptor antagonist deficient mice

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Abstract

The intestinal microbiome is perturbed in patients with new-onset and chronic autoimmune inflammatory arthritis. Recent studies in mouse models suggest that development and progression of autoimmune arthritis is highly affected by the intestinal microbiome. This makes modulation of the intestinal microbiota an interesting novel approach to suppress inflammatory arthritis. Prebiotics, defined as non-digestible carbohydrates that selectively stimulate the growth and activity of beneficial microorganisms, provide a relatively non-invasive approach to modulate the intestinal microbiota. The aim of this study was to assess the therapeutic potential of dietary supplementation with a prebiotic mixture of 90% short-chain galacto-oligosaccharides and 10% long-chain fructo-oligosaccharides (scGOS/lcFOS) in experimental arthritis in mice. We here show that dietary supplementation with scGOS/lcFOS has a pronounced effect on the composition of the fecal microbiota. Interestingly, the genera *Enterococcus* and *Clostridium* were markedly decreased by scGOS/lcFOS dietary supplementation. In contrast, the family Lachnospiraceae and the genus *Lactobacillus,* both associated with healthy microbiota, increased in mice receiving scGOS/lcFOS diet. Furthermore, we observed a significant increase in the bone mineral density in mice upon dietary supplementation with scGOS/lcFOS for 8 weeks. However, the scGOS/lcFOS induced alterations of the intestinal microbiota did not induce significant effects on the intestinal and systemic T helper cell subsets and were not sufficient to reproducibly suppress arthritis in mice. Altogether, this study suggests that dietary scGOS/lcFOS supplementation is able to promote presumably healthy gut microbiota and improve bone mineral density, but not inflammation, in arthritis-prone mice.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation and progressive destruction of cartilage and bone. Inflammatory cells such as T cells, B cells and macrophages accumulate in the inflamed joint, which results in synovitis and tissue destruction [1]. Although the exact etiology is unknown, RA is considered to be driven by genetic as well as environmental factors [1]. Several recent studies have shown that the composition of intestinal microbiota is perturbed in patients with new-onset as well as chronic RA [2-5]. This suggests that the microbiome may be an environmental factor that can influence the development of RA.

RA patients have increased levels of T helper-17 (Th17) cells in their peripheral blood mononuclear cells [6]. These Th17 cells are considered to be a major pathogenic mediator in RA, as these cells produce IL-17, a potent inducer of matrix metalloproteinases and proinflammatory cytokines such as interleukin-(IL)6 and IL-8. [6-9]. In addition, regulatory T (Treg) cells, which normally downregulate inflammation, were shown to have decreased suppressive activity in RA patients [10]. Intestinal microbiota strongly influences immune homeostasis and by altering the Th17/Treg cell balance the development of autoimmune diseases in mice [11-14]. Several studies have shown that development and severity of spontaneous arthritis in K/BxN and IL-1 receptor antagonist deficient (IL-1Ra$^{-/-}$) mice is strongly reduced in germ-free (GF) mice [11, 15, 16]. In addition, colonizing arthritis prone SKG mice with *Prevotella*-dominated microbiota of RA patients resulted in increased intestinal Th17 levels and aggravated arthritis development compared with mice receiving microbiota of healthy controls [17]. Furthermore, colonizing mice with the human gut commensal *Prevotella histicola* suppressed Th17 responses and the development of inflammatory arthritis after immunization with collagen type II [14]. These observations suggest that the intestinal microbiota plays an important role in the development of autoimmune arthritis, which makes modulation of the intestinal microbiota an interesting novel approach to suppress autoimmunity.

Prebiotics, defined as non-digestible carbohydrates that selectively stimulate the growth and activity of beneficial microorganisms, provide a relatively non-invasive approach to modulate the intestinal microbiota [18]. Dietary supplementation with a prebiotic mixture of 90% short-chain galaco-oligosaccharides and 10% long-chain fructo-oligosaccharides (scGOS/lcFOS) is known to particular promote the growth of beneficial bacteria such as bifidobacteria and lactobacilli [19-21]. In addition, several animal and clinical studies demonstrated that dietary supplementation with scGOS/lcFOS suppresses acute allergic symptoms, a process dependent on the induction of Treg cells [22-26]. Furthermore, multiple studies showed a beneficial effect of scGOS/lcFOS on bone mineral density [27, 28]. Something which could be beneficial in the context of RA, as bone mineral

density has been shown to be reduced in RA patients [29, 30].
The aim of the current study was to assess the efficacy of microbiota modulation using scGOS/lcFOS as a therapeutic approach for T cell-dependent autoimmune experimental arthritis in IL-1Ra$^{-/-}$ mice, which develop spontaneous arthritis due to excessive IL-1 receptor signaling [31]. We recently reported that IL-1Ra deficiency results in reduced diversity and richness, and causes specific taxonomic alterations characterized by increased *Helicobacter spp.* and decreased *Ruminococcus* spp. and *Prevotella* ssp., which specifically induces Th17 differentiation in intestinal lamina propria [16]. In addition, tobramycin-induced alterations of commensal intestinal microbiota suppressed arthritis in IL-1Ra$^{-/-}$ mice [16].
In this study we describe a significant increase in the bone mineral density after mice were on a diet supplemented with 5% scGOS/lcFOS for 8 weeks. Using high-throughput 16S rRNA marker gene sequencing, we here show that dietary supplementation with scGOS/lcFOS had a pronounced effect on the composition of the fecal microbiota. However, scGOS/lcFOS-induced alterations of the intestinal microbiota did not induce any significant beneficial effects on the intestinal and systemic T helper cell subsets and were unable to reproducibly suppress arthritis.

Materials and methods

Mice
IL-1Ra deficient mice on BALB/c background were kindly provided by Dr. M. Nicklin (Sheffield, England). The mice were housed in filter-top cages under specific pathogen-free conditions and the water and food were provided *ad libitum*. Age- and gender-matched littermates were used in all experiments, the average age at the start of the experiments was 8 weeks. Development of arthritis was scored macroscopically by two blinded observers using an arbitrary scoring system as follows; 0, no redness and swelling; 0.25, slight redness; 0.5, slight redness and swelling; 0.75-1, mild redness and swelling; 1.25-1.5, moderate redness and swelling; 1.75-2, severe redness and swelling. Only hind paws were scored, because arthritis development in the front paws is rare in this model [32]. Mice were randomly assigned to a group when reaching a score between 0.5-1. All animal procedures were approved by the ethics committee of the Radboud University Medical Center and were performed according to the appropriate codes of practice (approval number RU-DEC2010-082).

Prebiotic diet
The groups either received standard AIN-93 synthetic feed control diet or a diet supplemented with a mixture of scGOS (Vivinal GOS, Borculo Domo, Zwolle, The

Netherlands) and lcFOS (Raftiline HP, Orafti, Wijchen, The Netherlands) at a ratio of 9:1. The experimental diets contained either 1%, 2.5% or 5% scGOS/lcFOS added to standard AIN-93 synthetic feed (Research Diet Services, Wijk bij Duurstede, The Netherlands). The mice stayed on their respective diets for 8-10 weeks.

Microbiota sequencing and data analysis

After 8 weeks of dietary intervention, feces were collected and fecal bacterial DNA was isolated using phenol/chloroform-based extraction method combined with bead-beating [33]. Sequencing was performed by DNAVision (Charleroi, Belgium) on a Roche 454 GS-FLX System using 16S rRNA bar-coded primers targeting the V5-V6 conserved DNA regions (forward primer 784F: 5'-AGGATTAGATACCCTGGTA-3', reverse primer 1061R: 5'-CRRCACGAGCTGACGAC-3') [34]. For gene sequence analysis, a customized workflow based on Quantitative Insights Into Microbial Ecology (QIIME version 1.2) was adopted (http://qiime.org/) [35]. Settings recommended in QIIME 1.2 tutorial were applied. Additionally, reads were filtered for chimeric sequences using Chimera Slayer as described before [36]. Operational taxonomic unit (OTU) clustering was performed with settings as recommended by QIIME [37] using an identity threshold of 97%. The Ribosomal Database Project classifier version 2.2 was used for taxonomic classification [38]. Hierarchical clustering of samples was performed using the average distances between samples with weighted UniFrac as distance measure as implemented in QIIME. For statistical analysis and generation of figures, QIIME implemented R-packages, SciPy [39] (www.Scipy.org), Graphpad Prism version 5.0, and Microsoft® Office Excel® 2007 were adopted.

Histology

For histological assessment of arthritis, total ankle joints were isolated and fixed in 4% formaldehyde for 4 days, thereafter decalcified in 5% formic acid and embedded in paraffin. Tissue sections of 7µm were stained using Haematoxylin & Eosin to study synovial inflammation, chondrocyte death and cartilage and bone erosion. Safranin O staining was performed on the sections to determine proteoglycan depletion. Each parameter was scored on a scale from 0-3 in a blinded manner.

Lymphocyte isolation

Mice were sacrificed by cervical dislocation, immediately followed by isolation of the popliteal lymph nodes (pLN) and small intestine (SI). pLNs were disrupted on a 70 µm cell strainer, and the cells were collected in RPMI-1640 (Gibco; Invitrogen) supplemented with 10% FCS and gentamycin (50mg/l, Centrafarm). The SI was placed in ice-cold PBS and mesenteric fat and Peyer's patches were removed. This was followed by incubation with 33 mM EDTA on ice for 30 minutes

to remove epithelial cells, and subsequent digestion with 1 mg/ml collagenase-D (Roche) and 10 µg/ml DNAse I (Sigma) at 37 ^{o}C for three cycles of 15 minutes. Lamina propria lymphocytes (LPLs) were then harvested at the interphase of a 40:80% Percoll gradient (Sigma), washed thoroughly and stimulated and stained as described below.

Flow cytometry

LPLs and pLN cells stimulated for 4 hours with PMA (50 ng/ml; Sigma), ionomycine (1 µg/ml; Sigma), and the Golgi-traffic inhibitor Brefeldin (1 µl/ml; BD Biosciences). Cells were stained with anti-CD3-PE (BD pharmingen) or anti-CD3-APC (eBioscience) and anti-CD4-APC (Biolegend) or anti-CD4-FITC (BD pharmigen). Next, the cells were fixed and permeabilized using fixation/permeabilization buffer (eBioscience). For intracellular staining the cells were incubated in permeabilization buffer (eBioscience) containing anti-IL-17-FITC (Biolegend), anti-IFNγ-FITC (BD Pharmingen), anti-IL-4-PE (BD pharmigen) or Foxp3-FITC (eBioscience). An appropriate isotype matched control antibody was used in all FACS analyses. Cells were analyzed on a FACS Calibur using the CellQuest software (BD biosciences). Results were analyzed with FlowJo version 7.6.5.

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

Tissues were homogenized using a MagNA Lyser instrument (Roche). RNA was isolated in TRIzol reagent (Sigma) as described before [15]. Quantitative real-time PCR (qRT-PCR) was performed using the StepOne System (Applied Biosystems) using the SYBR green Master Mix (Applied Biosystems). Primer sequences were as follows: for GAPDH (House-keeping gene), 5'-GGCAAATTCAACGGCACA-3' (forward) and 5'-GTTAGTGGGGTCTCGCTCTG-3' (reverse); for T-bet 5'-CAACA ACCCCTTTGCCAAAG-3' (forward) 5'-TCCCCCAAGC AGTTGACAGT-3' (reverse); for RORγt 5'-CTGTCCTGGGCTACCCTACTGA-3' (forward) and 5'-AAGGGATCAC TTCAATTTGTGTTCTC-3' (reverse); for FoxP3 5'-AGGAGAAGCTGGGAGCTATGC-3' (forward) and 5'-GGTGGCTACGATTGCAGCAA-3' (reverse).

Dual-energy X-ray absorptiometry (DEXA) scanning

To assess the effect of scGOS/lcFOS on bone mineral density, dual-energy X-ray absorptiometry (DEXA, Lunar PIXImus) scanning was performed after 10 weeks of treatment. The mice were anesthetized for the duration of the procedure by exposure to 2.5% isoflurane-oxygen gas via a nose cone. One scan per mouse was performed and bone mineral density (g/cm^2) was calculated with PIXImus software. The head was excluded from the calculations using a manual region of interest.

Statistics

Differences in the relative abundance of bacterial taxa between treatment groups were evaluated using Mann-Whitney U test. We corrected for multiple testing using the Benjamini and Hochberg procedure with false discovery rate (FDR) set at 25%, and differences with a *p*-value < 0.05 which passed the FDR test were considered statistically significant. Kruskal-Wallis with a Dunn's post test was used to compare cell levels, arthritis histology scores, gene-expression and bone mineral density between treatment groups. For arthritis scores, two-tailed Mann-Whitney U test was performed for area under the curve.

Results

Prebiotic diet containing scGOS/lcFOS alters the composition of intestinal microbiota in IL-1Ra$^{-/-}$ mice

To determine the effect of a prebiotic diet containing scGOS/lcFOS on the intestinal microbiota, IL-1Ra$^{-/-}$ mice were fed either a control diet, or a diet containing 1 or 2.5% scGOS/lcFOS for 8 weeks. The diet was well tolerated and did not cause any growth retardation or weight loss (data not shown). 16S rRNA marker gene pyrosequencing was performed on DNA from fecal samples collected after 8 weeks of intervention to identify changes in the intestinal microbiota. The average sequencing depth, total number of reads and operational taxonomic units (OTU) were not affected by the scGOS/lcFOS diet and remained comparable between the experimental groups (Supplementary Table1).

Furthermore, we did not observe any significant changes in the number of observed species, Chao1 index, Shannon index or phylogenetic distance whole tree metric (Supplementary Figure 1A-C). In addition, principal coordinates analysis (PCoA) based on weighted UniFrac distances showed no clear differences between the different groups (Supplementary Figure 1D). Although we did not observe any significant effect on bacterial richness and diversity, the scGOS/lcFOS diet significantly altered the composition of the intestinal microbiota. A prominent effect observed in the 2.5% scGOS/lcFOS fed mice compared to the control group was a highly significant increase in the family Lachnospiraceae (Figure 1 and Supplementary Table 2). However, the resolution of the 16S gene pyrosequencing was not sufficient to identify the genera within the family Lachnospiraceae that were increased in the 2.5% scGOS/lcFOS fed mice (Figure 1 and Supplementary Table 2).

A significant increase in the genus *Lactobacillus* was observed for mice receiving the 2.5% scGOS/lcFOS, corroborating results observed previously by Vos *et al.* (Figure 1 and Supplementary Table 2) [20]. The genus *Barnesiella* (family Porphyromonadaceae) was increased as well in the 2.5% scGOS/lcFOS group (Figure 1 and Supplementary Table 2), although still represented a low abundant taxon. A significant near complete elimination of bacteria belonging to the genus

Turicibacter (family Erysipelotrichaceae) was observed in the 2.5% scGOS/lcFOS fed mice (Figure 1 and Supplementary Table 2). In addition, the genera *Oscillibacter* (family Ruminococcacea), *Enterococcus* (family Enterococcaceae), *Streptococcus* (family Streptococcaceae), *Lactococcus (*family Streptococcaceae) and *Clostridium (*family *Clostridiaceae)* were significantly decreased in the 2.5% scGOS/lcFOS group (Figure 1 and Supplementary Table 2), although none of these taxa were highly dominant among the microbiota. None of the observed differential abundant taxa in the 2.5% scGOS/lcFOS group were found to be significant in the group receiving the 1% scGOS/lcFOS diet; however, the fold changes for the 1% scGOS/lcFOS group correlated significantly with the change for the 2.5% scGOS/lcFOS group (spearman rank test: rho 0.45, p-value 0.003). Altogether, these data show that a 2.5% scGOS/lcFOS diet alters the composition of the intestinal microbiota.

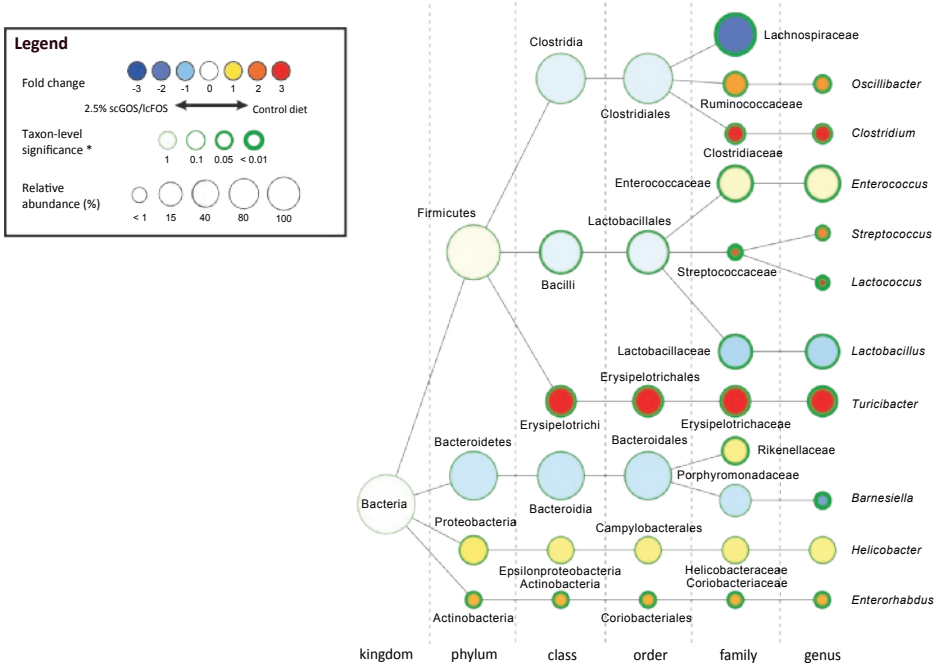


Figure 1. Prebiotic diet containing scGOS/lcFOS significantly alters the composition of intestinal microbiota of IL-1Ra$^{-/-}$ mice. Phylogenetic tree created by Cytoscape software showing specific changes in intestinal microbial community at different taxonomic levels in the mice fed 2.5% scGOS/lcFOS diet compared to mice fed a control diet. Nodes represent taxa, and the size of each node represents its relative abundance. The color blue indicates an increase in the 2.5% scGOS/lcFOS fed mice compared to control mice, while the color red indicates an decrease in the 2.5% scGOS/lcFOS fed mice. The thickness of the green border indicates the degree of statistical significance by Mann-Whitney U test, uncorrected.

Prebiotic diet containing 5% scGOS/lcFOS diet significantly improves bone mineral density

It has previously been described that scGOS/lcFOS diet can increase intestinal mineral absorption from diet and thereby improve bone mineral density in rats [27, 28]. Therefore, we performed DEXA scanning to measure bone mineral density in our mice. This revealed that a prebiotic diet containing 5% scGOS/lcFOS significantly improves the overall bone mineral density of IL-1Ra$^{-/-}$ mice (Figure 2A). The bone mineral content (BMC) also tended to be increased in the 5% scGOS/lcFOS treated mice; however, this increase was statistically not significant (Figure 2B). This finding indicates that the scGOS/lcFOS diet has a beneficial effect on bone mineral density during experimental arthritis.

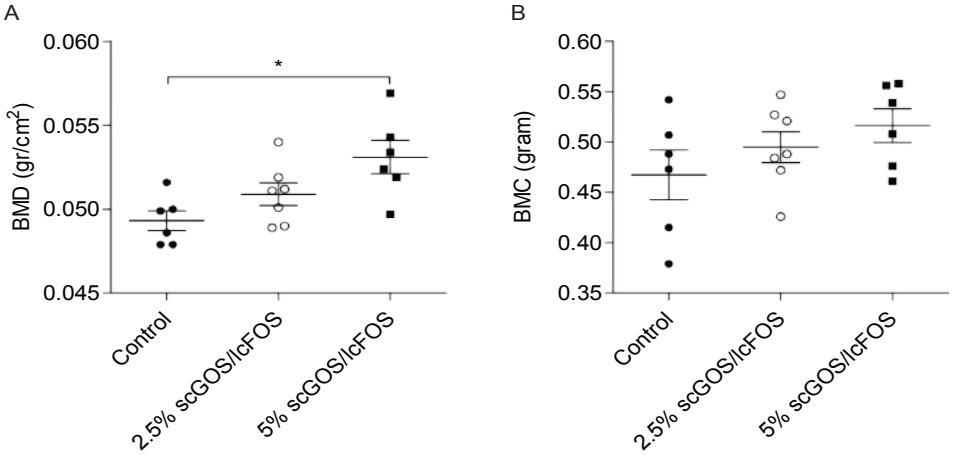


Figure 2. Prebiotic scGOS/lcFOS diet improves the overall bone mineral density in arthritic IL-1Ra deficient mice. (A) Bone mineral density (BMD) and (B) Bone mineral content (BMC) of arthritic IL-1Ra$^{-/-}$ mice. Dual-energy X-ray absorptiometry (DEXA) scanning was performed after 10 weeks of dietary treatment with either 2.5% or 5% scGOS/lcFOS. *p<0.05 by Kurskal-Wallis with Dunn's post test.

Treatment of arthritic IL-1Ra$^{-/-}$ mice with scGOS/lcFOS diet has no effect on the progression of experimental arthritis

To determine the efficacy of scGOS/lcFOS in the treatment of joint inflammation as well as cartilage and bone destruction during experimental arthritis, IL-1Ra$^{-/-}$ mice with ongoing arthritis under conventional microbial status were orally fed a control diet or a diet containing 1% or 2.5% scGOS/lcFOS for 8 weeks. The severity of arthritis over time was comparable between the group receiving the 1% scGOS/lcFOS diet and the control group. The mice in the 2.5% scGOS/lcFOS group showed a trend toward reduced arthritis severity scores over the entire 8-week study period; however, this effect was not significant (p=0.094; Figure 3A). Histological examination of the ankle joints confirmed this lack of therapeutic efficacy of the scGOS/lcFOS diet and, revealed no significant effects on inflammation, bone and cartilage damage (Figure 3B). To determine the effect

of the different scGOS/lcFOS diets on the local T cell response, we determined the gene expression of the transcription factors *Tbet*, *RORγt* and *FoxP3* (relevant for differentiation of Th1, Th17 and Tregs, respectively) in pLNs, which drain the arthritic ankle joint. The gene expression of *Tbet* and *RORγt* was significantly reduced in pLNs of the mice which received the 2.5% scGOS/lcFOS diet compared to the control mice (Figure 3C-D). However, the expression of FoXP3 was not affected by the diet (Figure 3E).

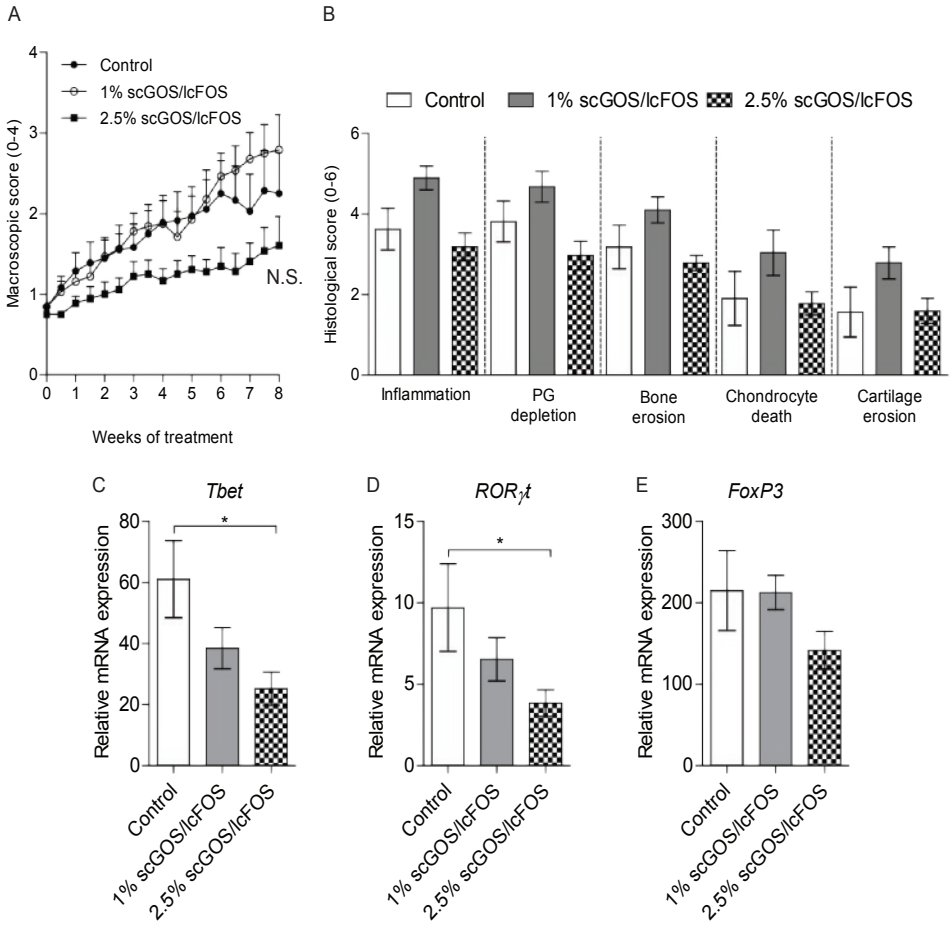


Figure 3. Oral treatment of arthritic IL-1Ra$^{-/-}$ mice with prebiotic scGOS/lcFOS has no effect on the progression of arthritis. (A) Arthritis severity scores (0-2 per paw) of IL-1Ra$^{-/-}$ mice fed a control diet or a diet containing either 1% or 2.5% scGOS/lcFOS for 8 weeks. (B) Histological scores of synovial inflammation, proteoglycan (PG) depeletion, bone erosion, chondrocyte (chond.) death and cartilage erosion. Data shown mean + SEM of 8-9 mice per group. Treatment started when mice had a score of 0.75-1. (C-E) Gene expression of Tbet (C), RORγt (D) and FoxP3 (E) in joint draining lymph nodes of arthritic IL-1Ra$^{-/-}$ mice fed a control diet (n=5) or a diet containing either 1% (n=5) or 2.5% (n=8) scGOS/lcFOS. Relative mRNA expression is shown as $2^{-\Delta Ct}$ *10000, corrected for GAPDH. *p<0.05 by Kurskal-Wallis with Dunn's post test.

Aiming to maximize the observed effects of the dietary scGOS/lcFOS supplement, we replicated the experiment with IL-1Ra$^{-/-}$ mice receiving either 2.5% or 5 % scGOS/lcFOS supplemented diets. However, this study showed no effect of the prebiotic diet on arthritis scores at any dose (Supplementary Figure 2). In addition, we isolated cells from the draining lymph nodes and performed flow cytometric analysis. This analysis showed no effect of the different scGOS/lcFOS doses on the abundance of Th1, Th2, Th17 and Treg cells in pLNs (Supplementary Figure 3A-D). Based on these data, we conclude that scGOS/lcFOS-induced alterations of the intestinal microbiota were not sufficient to significantly alter the joint-associated T helper cells subsets and reproducibly suppress arthritis.

scGOS/lcFOS diet has no effect on intestinal T helper cell subsets in IL-1Ra$^{-/-}$ mice

Intestinal microbiota are known to greatly influence the balance between pro-inflammatory and regulatory mucosal T cell responses [40]. Considering the observed effects of scGOS/lcFOS on the intestinal microbiota, we investigated the gene expression of the transcription factors *Tbet*, *RORγt* and *FoxP3* relevant for differentiation of Th1, Th17 and Tregs, respectively, in ileum, mesenteric lymph nodes (mLN) and spleen of IL-1Ra$^{-/-}$ mice fed 1% and 2.5% scGOS/lcFOS diet. We observed no effect of the scGOS/lcFOS diet on expression levels of these genes in any of the tissues we tested (Supplementary Figure 4A-C). However, *FoxP3* mRNA expression in the colon of 2.5% and 5% scGOS/lcFOS fed mice was slightly, but not significantly, increased compared to mice on a control diet (Supplementary Figure 4D).

In addition, we analyzed the effect of the 2.5% and 5% scGOS/lcFOS diet on T helper cell subset in the small intestine lamina propria with flow cytometry. The small intestine lamina propria (SI-LP) of mice on the 5% scGOS/lcFOS diet contained slightly increased percentages of Th17, Th1 and Tregs, however these effect failed to reach statistical significance (Supplementary Figure 5A-C). In contrast, the percentage of IL-4 producing Th2 cells present in the SI-LP showed a non-significant reduction in the 5% scGOS/lcFOS group compared to the control group (Supplementary Figure 5D). We conclude from these data that although a scGOS/lcFOS diet significantly affected the intestinal microbiome, it did not alter mucosal T helper cell subsets in intestinal lamina propria.

Discussion

Recent developments in the fields of microbiome research and immunology have shown that intestinal microbiota play a critical role in the maintenance of immune homeostasis [41-43]. Therefore, modulation of the intestinal microbiota may offer an interesting novel approach to suppress autoimmunity. In this study, we assessed the efficacy of microbiota modulation using a specific prebiotic mixture as a therapeutic approach in experimental arthritis.

For the study presented here we used IL-1Ra$^{-/-}$ mice which spontaneously develop arthritis due to excessive IL-1 receptor signaling [31]. We have previously shown that arthritis development in these mice is highly dependent on the intestinal microbiome as arthritis is strongly attenuated under germ-free conditions [15, 16]. In the current study we show that a 2.5% scGOS/lcFOS dietary supplementation had no significant effects on the microbial richness or diversity in IL-1Ra$^{-/-}$ mice; however, it resulted in an altered composition of the intestinal microbiota. This was most notably characterized by a significant increase in Lachnospiraceae spp. and *Lactobacillus* ssp.. Members of the family Lachnospiraceae have recently been linked to alleviation of experimental encephalomyelitis [44]. It was hypothesized that the increase in Lachnospiraceae resulted in an increased production of intestinal butyrate [44]. Butyrate is a short chain fatty acid known to induce differentiation of Treg cells and reduce colonic inflammation [45-48]. In addition, a recent study showed that the composition of microbiota prior to arthritis onset differs between the collagen induced arthritis (CIA)-susceptible and CIA–resistant mice [49]. This study found that Lachnospiraceae was more abundant in CIA-resistant mice, while Lactobacillaceae was more abundant in CIA-susceptible mice [49]. Furthermore, Lachnospiraceae was found to be decreased in gut microbiota of psoriatic arthritis patients [50]. In our study, however, the increase in Lachnospiraceae did not result in a significant suppression of IL-1Ra$^{-/-}$ arthritis.

In addition, it has been reported that *Clostridium difficile*-infected mice with a microbiota dominated by Lachnospiraceae developed a milder disease [51]. Another clinical study showed that the presence of Lachnospiraceae was associated with lower risk of *Clostridium difficile* infection in adult recipients of allogeneic hematopoietic stem cells transplantation [52]. Furthermore, imbalances observed in the gut microbiota of inflammatory bowel disease patients was characterized by reduced abundance of Lachnospiraceae [53]. These studies suggest a beneficial role for Lachnospiraceae in gut health and protection against pathogens.

In mice, scGOS/lcFOS dietary supplementation also resulted in an increased prevalence of fecal Bifidobacteria and lactobacilli [20]. In accordance with these studies, we observed an increase of 2.83% in *Lactobacillus* in the 2.5% scGOS/lcFOS fed mice in comparison to control diet, however bifidobacteria were absent in our IL-1Ra$^{-/-}$ mice and could therefore not be affected in our study. Added to infant formulas, scGOS/lcFOS has been described to stimulated the growth of bifidobacteria and lactobacilli and reduce the numbers of pathogenic bacteria [19, 54, 55]. In addition, a recent paper described that infants receiving scGOS/lcFOS supplemented formula showed increased *Bifidobacterium* and decreased Clostridium and *Lachnospiraceae* [56]. In agreement with this study *Clostridium* was decreased in the scGOS/lcFOS treated mice in our studies; however, we observed a strong increase in the family *Lachnospiraceae*. Therefore, the effects

observed in our study differ markedly from the effects observed in infants, suggesting that the effect of scGOS/lcFOS depends on the host and endogenous microbiome at start of treatment

In this study we show that bone mineral density is increased in mice fed a diet supplemented with 5% scGOS/lcFOS. This is in agreement with previous studies which showed that a scGOS/lcFOS mixture increases mineral absorption and bone mineral density in rats [27, 28]. Similar to our current study, these studies observed an increase in the abundance of *Lactobacillus* in the rats receiving the scGOS/lcFOS supplemented diet [28]. In addition, those studies reported a reduction in cecal pH values [27, 28]. It was therefore hypothesized that the scGOS/lcFOS diet increased the production of organic acids (short chain fatty acids and lactic acids) by lactic acid bacteria such as *Lactobacillus*, which lowers the pH and thereby improves mineral absorption [27]. However, since we do not have 16S data of mice receiving 5% scGOS/lcFOS we do not know which bacteria are responsible for this effect in our study. Altogether, the significant improvement of the bone mineral density suggests that the scGOS/lcFOS prebiotic mixture has beneficial effects on the bone in the context of arthritis.

We previously showed that the aberrant microbiota in IL-1Ra$^{-/-}$ mice specifically induced IL-17 production by intestinal lamina propria lymphocytes, an effect that could be transferred to wild-type mice by fecal microbiota [16]. Previous studies showed that a scGOS/lcFOS containing diet enhanced the percentage of Th1 cells and tended to reduce Th2 response in mice [57, 58]. In another study it was shown that suppression of the allergic responses by scGOS/lcFOS depends on the presence of CD25+ Tregs [22, 23]. Furthermore, lactobacilli are thought to induce Treg differentiation by modulating dendritic cell function [59]. In addition, butyrate produced by Lachnospiraceae could also induce Treg differentiation [45]. This suggests that a scGOS/lcFOS diet and subsequent increase in *Lactobacillus* and Lachnospiraceae could cause an anti-inflammatory shift in Th cell responses. However, analysis of the intestinal lamina propria lymphocytes with flow cytometry in our study did not show any effect on Th cell subsets. This suggests that despite the effect of scGOS/lcFOS on the intestinal microbiota, the diet did not result in modulation of the intestinal immune response in IL-1Ra$^{-/-}$ mice. Excessive IL-1 signaling is known to downregulate TGF-β induced Foxp3 expression and enhance Th17 differentiation [60]. The lack of modulation of Th cells and arthritis development in our studies could be due to the enhanced IL-1 signaling in IL-1Ra deficient mice, overruling the immune suppressive effects of the scGOS/lcFOS modulated microbiota.

Another possibility is that the specific microbiota modulated by the scGOS/lcFOS diet were not relevant to the ongoing inflammatory processes and that the Th17-driving bacteria were not affected. We recently demonstrated that, IL-1Ra deficiency reduces the intestinal microbial diversity and richness, and causes specific alterations in composition of the intestinal microbiota [16].

The taxonomic alterations in IL-1Ra$^{-/-}$ mice were characterized by overrepresentation of the genera *Helicobacter*, *Rikenella, Butyricimonas* and *Streptococcus,* while the genera *Prevotella, Parasutterella, Xylanibacter, Ruminococcus, and Barnesiella* were underrepresented in the IL-1Ra$^{-/-}$ mice compared to the WT mice [16]. Interestingly, in the 2.5% scGOS/lcFOS fed mice *Streptococcus* were decreased and *Barnesiella* was increased compared to the control group. This might suggest that a 2.5% scGOS/lcFOS diet can partly restore the dysregulated microbiota of IL-1Ra$^{-/-}$ mice. Treatment of IL-1Ra$^{-/-}$ mice with tobramycin significantly reduced arthritis severity and resulted in a near-complete elimination of *Helicobacter* and a highly significant reduction of *Clostridium* [16]. In this current study, 2.5% scGOS/lcFOS diet did not have a strong effect on *Helicobacter*, as only a small non-significant decrease was observed in the 2.5% scGOS/lcFOS treated group (3.13% in control group vs. 2.08% in 2.5% scGOS/lcFOS group). However, scGOS/lcFOS treatment did significantly reduce *Clostridium* abundance (Figure 1), which was also one of the genera significantly affected by tobramycin treatment. This suggests that bacteria which contribute to the progression of arthritis in IL-1Ra$^{-/-}$ mice are only partly affected by scGOS/lcFOS supplementation.

Conclusions

Prebiotics such as scGOS/lcFOS have potential benefits in providing nutrient sources to specific beneficial bacteria to promote a diverse and healthy gut microbiota. In our study, we observed an increase in *Lactobacillus* genus and Lachnospiraceae family after 8 weeks of dietary scGOS/lcFOS supplementation during arthritis. In addition, we found a beneficial effect of the scGOS/lcFOS diet on BMD in arthritic mice. However, despite these positive effects on bone and the microbiota composition, the scGOS/lcFOS diet did not induce a change in Th cell subsets or a reproducible therapeutic effect on the progression of autoimmune arthritis in IL-1Ra$^{-/-}$ mice. Altogether, this study suggests the ability of scGOS/lcFOS supplement to alter the gut microbiota into a more beneficial state and improving the bone mineral density.

References

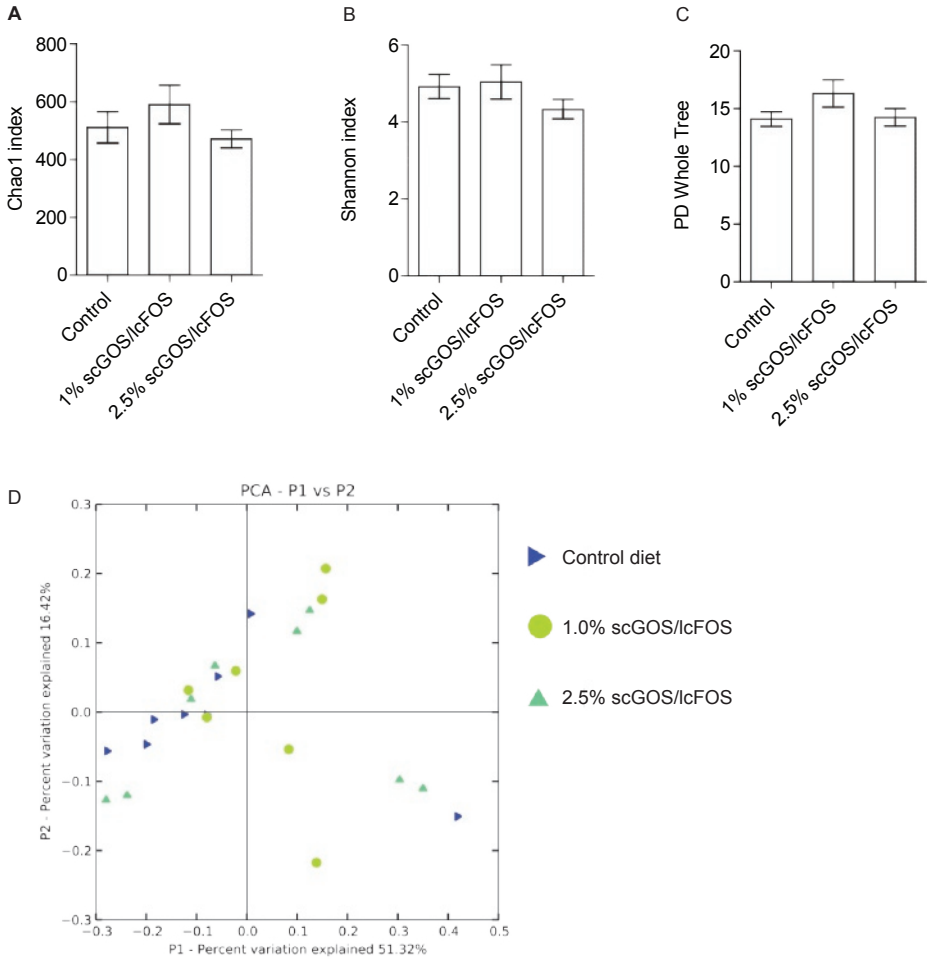
1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. The New England journal of medicine. 2011;365(23):2205-19.
2. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. eLife. 2013;2:e01202.
3. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nature medicine. 2015;21(8):895-905.
4. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis contributes to arthritis development via activation of autoreactive T cells in the intestine. Arthritis & rheumatology (Hoboken, NJ). 2016.
5. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome medicine. 2016;8(1):43.
6. van Hamburg JP, Asmawidjaja PS, Davelaar N, Mus AM, Colin EM, Hazes JM, et al. Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis and rheumatism. 2011;63(1):73-83.
7. Lubberts E. Th17 cytokines and arthritis. Semin Immunopathol. 2010;32(1):43-53.
8. Gaffen SL. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. Current rheumatology reports. 2009;11(5):365-70.
9. van den Berg WB, Miossec P. IL-17 as a future therapeutic target for rheumatoid arthritis. Nature reviews Rheumatology. 2009;5(10):549-53.
10. Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M, et al. Protein kinase C-theta mediates negative feedback on regulatory T cell function. Science (New York, NY). 2010;328(5976):372-6.
11. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
12. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. Journal of immunology (Baltimore, Md : 1950). 2009;183(10):6041-50.
13. Ochoa-Reparaz J, Mielcarz DW, Ditrio LE, Burroughs AR, Begum-Haque S, Dasgupta S, et al. Central nervous system demyelinating disease protection by the human commensal Bacteroides fragilis depends on polysaccharide A expression. Journal of immunology (Baltimore, Md : 1950). 2010;185(7):4101-8.
14. Marietta EV, Murray JA, Luckey DH, Jeraldo PR, Lamba A, Patel R, et al. Suppression of Inflammatory Arthritis by Human Gut-Derived Prevotella histicola in Humanized Mice. Arthritis & rheumatology (Hoboken, NJ). 2016;68(12):2878-88.
15. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. The Journal of clinical investigation. 2008;118(1):205-16.
16. Rogier R, Ederveen THA, Boekhorst J, Wopereis H, Scher JU, Manasson J, et al. Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis. Microbiome. 2017;5(1):63.
17. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine. Arthritis & rheumatology (Hoboken, NJ). 2016;68(11):2646-61.
18. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of nutrition. 1995;125(6):1401-12. gastroenterology and nutrition. 2002;34(3):291-5.

19. Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, et al. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. Journal of pediatric gastroenterology and nutrition. 2002;34(3):291-5.
20. Vos AP, Haarman M, van Ginkel JW, Knol J, Garssen J, Stahl B, et al. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2007;18(4):304-12.
21. Haarman M, Knol J. Quantitative real-time PCR analysis of fecal Lactobacillus species in infants receiving a prebiotic infant formula. Applied and environmental microbiology. 2006;72(4):2359-65.
22. Schouten B, van Esch BC, Hofman GA, Boon L, Knippels LM, Willemsen LE, et al. Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. The Journal of nutrition. 2010;140(4):835-41.
23. Schouten B, van Esch BC, Hofman GA, de Kivit S, Boon L, Knippels LM, et al. A potential role for CD25+ regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates. The British journal of nutrition. 2012;107(1):96-105.
24. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Archives of disease in childhood. 2006;91(10):814-9.
25. Schouten B, Van Esch BC, Kormelink TG, Moro GE, Arslanoglu S, Boehm G, et al. Non-digestible oligosaccharides reduce immunoglobulin free light-chain concentrations in infants at risk for allergy. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2011;22(5):537-42.
26. van der Aa LB, Heymans HS, van Aalderen WM, Sillevis Smitt JH, Knol J, Ben Amor K, et al. Effect of a new synbiotic mixture on atopic dermatitis in infants: a randomized-controlled trial. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2010;40(5):795-804.
27. Bryk G, Coronel MZ, Lugones C, Mandalunis P, Rio ME, Gualtieri AF, et al. Effect of a mixture of GOS/FOS(R) on calcium absorption and retention during recovery from protein malnutrition: experimental model in growing rats. European journal of nutrition. 2016;55(8):2445-58.
28. Bryk G, Coronel MZ, Pellegrini G, Mandalunis P, Rio ME, de Portela ML, et al. Effect of a combination GOS/FOS(R) prebiotic mixture and interaction with calcium intake on mineral absorption and bone parameters in growing rats. European journal of nutrition. 2015;54(6):913-23.
29. Lodder MC, de Jong Z, Kostense PJ, Molenaar ET, Staal K, Voskuyl AE, et al. Bone mineral density in patients with rheumatoid arthritis: relation between disease severity and low bone mineral density. Annals of the rheumatic diseases. 2004;63(12):1576-80.
30. Bugatti S, Bogliolo L, Vitolo B, Manzo A, Montecucco C, Caporali R. Anti-citrullinated protein antibodies and high levels of rheumatoid factor are associated with systemic bone loss in patients with early untreated rheumatoid arthritis. Arthritis research & therapy. 2016;18(1):226.
31. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. The Journal of experimental medicine. 2000;191(2):313-20.
32. Koenders MI, Devesa I, Marijnissen RJ, Abdollahi-Roodsaz S, Boots AM, Walgreen B, et al. Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice. Arthritis and rheumatism. 2008;58(11):3461-70.
33. Matsuki T, Watanabe K, Fujimoto J, Kado Y, Takada T, Matsumoto K, et al. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Applied and environmental microbiology. 2004;70(1):167-73.
34. Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PloS one. 2008;3(7):e2836.

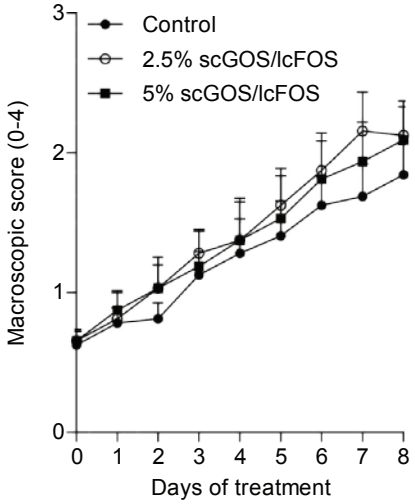
35. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 2010;7(5):335-6.
36. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome research. 2011;21(3):494-504.
37. QIIME. New default parameters for uclust OTU pickers https://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/2010 [updated December 17, 2010; cited 2016 May 25]. Available from: https://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/.
38. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic acids research. 2009;37(Database issue):D141-5.
39. SciPy.org. SciPy open-source software 2012 [25.05.16]. Available from: http://www.scipy.org/.
40. Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? Nature reviews Immunology. 2010;10(10):735-44.
41. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75-84.
42. Kamada N, Nunez G. Regulation of the immune system by the resident intestinal bacteria. Gastroenterology. 2014;146(6):1477-88.
43. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science (New York, NY). 2012;336(6086):1268-73.
44. Stanisavljevic S, Lukic J, Sokovic S, Mihajlovic S, Mostarica Stojkovic M, Miljkovic D, et al. Correlation of Gut Microbiota Composition with Resistance to Experimental Autoimmune Encephalomyelitis in Rats. Frontiers in microbiology. 2016;7:2005.
45. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5.
46. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science (New York, NY). 2013;341(6145):569-73.
47. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50.
48. Zimmerman MA, Singh N, Martin PM, Thangaraju M, Ganapathy V, Waller JL, et al. Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells. American journal of physiology Gastrointestinal and liver physiology. 2012;302(12):G1405-15.
49. Liu X, Zeng B, Zhang J, Li W, Mou F, Wang H, et al. Role of the Gut Microbiome in Modulating Arthritis Progression in Mice. Scientific reports. 2016;6:30594.
50. Scher JU, Ubeda C, Artacho A, Attur M, Isaac S, Reddy SM, et al. Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease. Arthritis & rheumatology (Hoboken, NJ). 2015;67(1):128-39.
51. Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut microbes. 2011;2(3):145-58.
52. Lee YJ, Arguello ES, Jenq RR, Littmann E, Kim GJ, Miller LC, et al. Protective Factors in the Intestinal Microbiome Against Clostridium difficile Infection in Recipients of Allogeneic Hematopoietic Stem Cell Transplantation. The Journal of infectious diseases. 2017;215(7):1117-23.

53. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(34):13780-5.
54. Knol J, Boehm G, Lidestri M, Negretti F, Jelinek J, Agosti M, et al. Increase of faecal bifidobacteria due to dietary oligosaccharides induces a reduction of clinically relevant pathogen germs in the faeces of formula-fed preterm infants. Acta paediatrica (Oslo, Norway : 1992) Supplement. 2005;94(449):31-3.
55. Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. Journal of pediatric gastroenterology and nutrition. 2005;40(1):36-42.
56. Wopereis H, Sim K, Shaw A, Warner JO, Knol J, Kroll JS. Intestinal Microbiota in Infants at High-risk for Allergy: Effects of Prebiotics and Role in Eczema Development. The Journal of allergy and clinical immunology. 2017.
57. Schouten B, van Esch BC, Hofman GA, van Doorn SA, Knol J, Nauta AJ, et al. Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. The Journal of nutrition. 2009;139(7):1398-403.
58. Vos AP, van Esch BC, Stahl B, M'Rabet L, Folkerts G, Nijkamp FP, et al. Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. International immunopharmacology. 2007;7(12):1582-7.
59. Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. The Journal of allergy and clinical immunology. 2005;115(6):1260-7.
60. Ikeda S, Saijo S, Murayama MA, Shimizu K, Akitsu A, Iwakura Y. Excess IL-1 signaling enhances the development of Th17 cells by downregulating TGF-beta-induced Foxp3 expression. Journal of immunology (Baltimore, Md : 1950). 2014;192(4):1449-58.

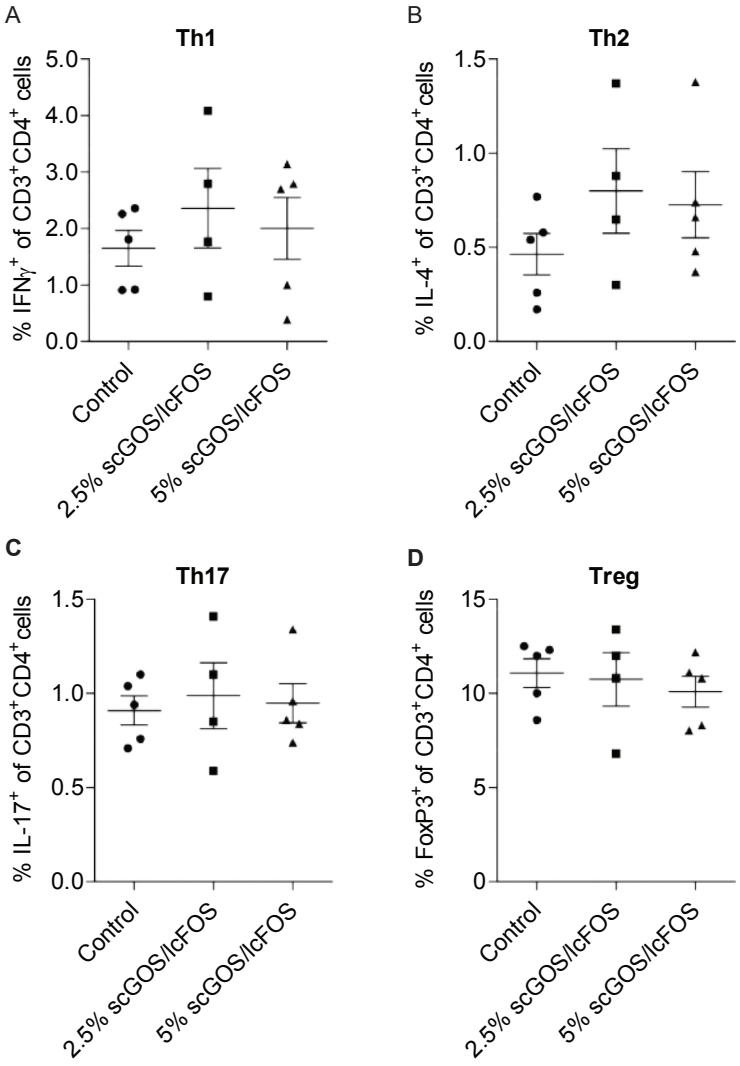
Supplementary material



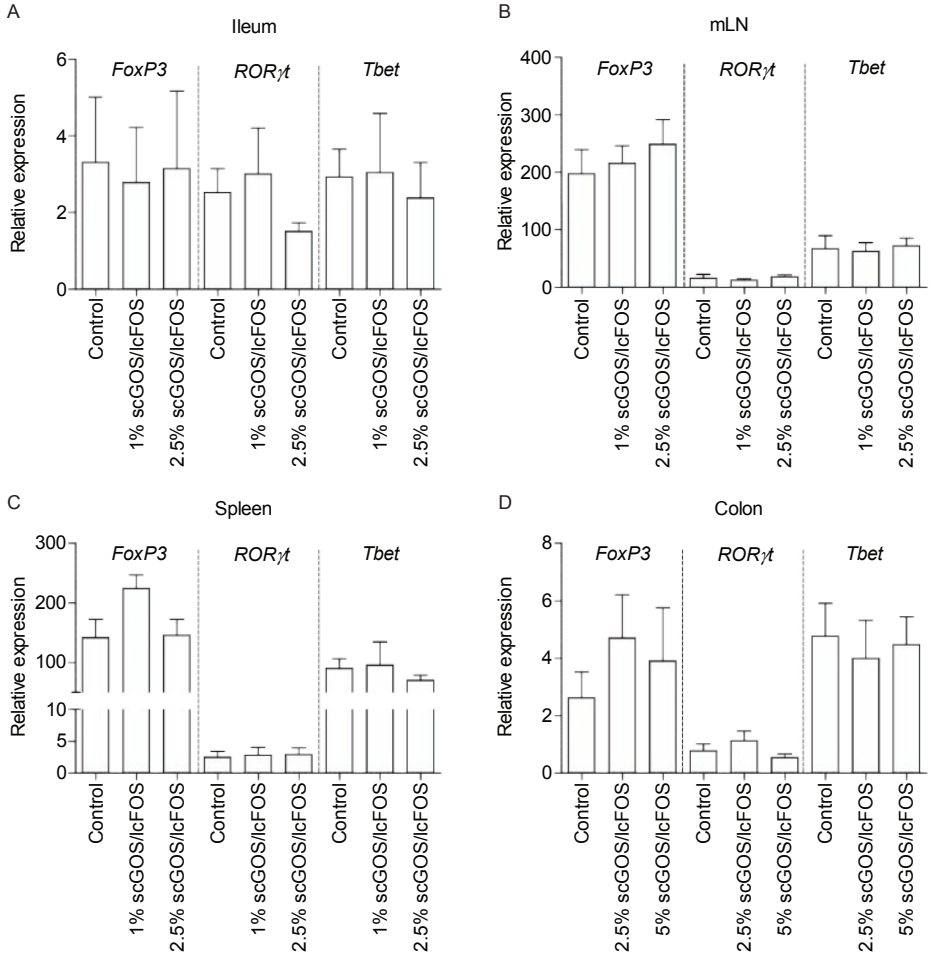
Supplementary Figure 1. Dietary supplementation with scGOS/lcFOS has no effect on bacterial richness and diversity. (A) Chao index1, (B) Shannon index, (C) PD whole tree are shown. (D) Principal coordinates analysis (PCoA) based on an unweighted UniFrac analysis of the intestinal microbial composition. The position and distance of data points indicates the degree of similarity in terms of both presence and relative abundance of bacterial taxonomies. Data (mean + SEM) represent 16S rRNA gene 454-pyrosequencing analysis of intestinal microbiota of of IL-1Ra$^{-/-}$ mice fed a control diet (n=8) or a diet containing either 1% (n=7) or 2.5% (n=8) scGOS/lcFOS for 8 weeks.



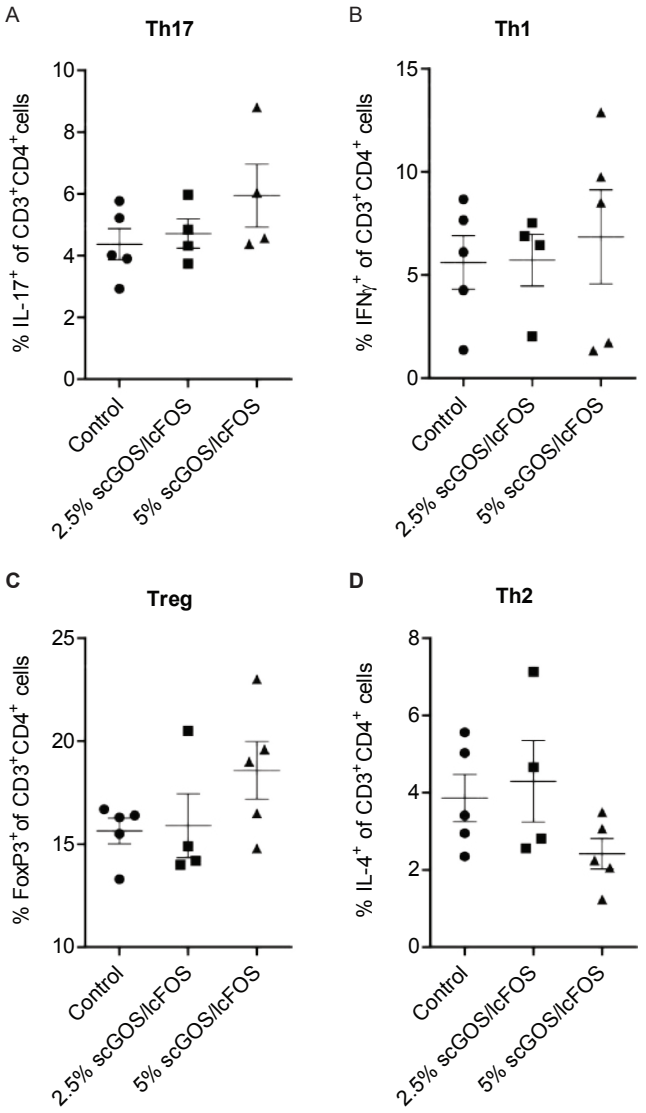
Supplementary figure 2. Treatment of arthritic IL-1Ra$^{-/-}$ mice with prebiotic scGOS/lcFOS has no effect on the progression of arthritis. Arthritis severity scores of IL-1Ra$^{-/-}$ mice fed a control diet or a diet containing either 2.5% or 5% scGOS/lcFOS for 8 weeks. Data shown mean + SEM of 8-9 mice per group. Treatment started when mice had a score of 0.75-1.



Supplementary figure 3. Diet containing scGOS/lcFOS has no effect on Thelper cell subsets in joint draining lymph nodes. Dot plots showing percentage of IFNγ^{+} Th1 (A) IL-4^{+} Th2 (B) IL-17^{+} Th17 (C) and FoxP3^{+} Treg cells among CD3^{+}CD4^{+} cells isolated from the joint draining lymph nodes of arthritic IL-1Ra$^{-/-}$ mice. The mice were on either 2.5% or 5% scGOS/lcFOS diet or were fed a control diet.



Supplementary figure 4. scGOS/lcFOS diet has no effect on Th cells subsets in IL-1Ra$^{-/-}$ mice. Gene expression of FoxP3, RORγt and Tbet in ileum (A), mesenteric lymph nodes (B), spleen (C) and colon (D) of IL-1Ra-/- mice fed a diet containing either 1% , 2.5% or 5% scGOS/lcFOS. Relative mRNA expression is shown as $2^{-\Delta Ct}$ *10000, corrected for GAPDH.



Supplementary figure 5. Intestinal T helper cells subsets not affected by scGOS/lcFOS containing diet. Dot plots showing percentage of IFNγ^{+} Th1 (A) IL-4^{+} Th2 (B) IL-17^{+} Th17 (C) and FoxP3^{+} Treg cells among CD3^{+}CD4^{+} cells isolated from the small intestine lamina propria of arthritic IL-1Ra$^{-/-}$ mice. The mice were on either 2.5% or 5% scGOS/lcFOS diet or were fed a control diet.

Supplementary Table 1. The average and total number of (assigned) reads and operational taxonomic units (OTU) per experimental group. In addition, the number and percentage of reads assigned to phylum or genus level are shown.

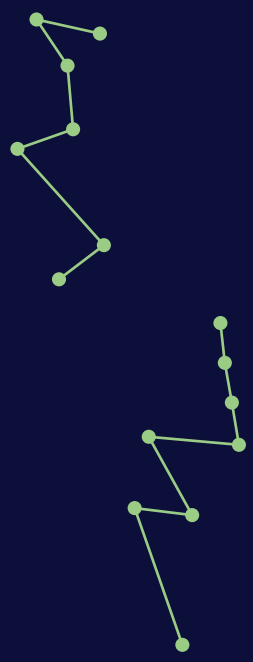
	Reads			OTU			Assigned at Phylum		Assigned at Genus		
Diet	**Average**	**SEM**	**Total**	**Average**	**SEM**	**Total**	**Total Reads**	**%**	**Total Reads**	**%**	**Group Size**
Control	8555	578	68441	545	55	4362	67350	98.4%	33082	48.3%	n = 8
1.0% scGOS/ lcFOS	6544	644	45808	498	41	3487	44929	98.1%	22674	49.5%	n = 7
2.5% scGOS/ lcFOS	6796	298	54364	422	23	3379	53845	99.0%	22239	40.9%	n = 8

Supplementary Table 2. Prebiotic diet containing scGOS/lcFOS alteres the composition of intestinal microbiota IL-1Ra$^{-/-}$ mice. Relative abundance on family and genus level in IL-1Ra$^{-/-}$ mice fed either a control diet or a diet containing 1.0% or 2.5% short-chain galaco-oligosaccharides / fructo-oligosaccharides (scGOS/lcFOS). Significant alterations by Mann-Whitney U (MWU) after Benjamini-Hochberg correction (FDR) for multiple testing are in bold. The color blue indicates an increase in the treatment group compared to the control group, while the color red indicates a decrease.

		Relative abundance			Fold change	
Taxon level	**Taxon name**	**Control diet**	**1.0%**	**2.5%**	**Control diet vs. 1.0%**	**Control diet vs. 2.5%**
genus	Turicibacter	8.37%	0.02%	0.01%	-8.92	**-9.52**
family	Erysipelotrichaceae	8.57%	0.11%	0.14%	-6.30	**-5.93**
genus	Clostridium	2.27%	0.00%	0.12%	na	**-4.23**
family	Clostridiaceae	2.48%	0.00%	0.13%	na	**-4.23**
genus	Lactococcus	0.10%	0.05%	0.01%	-1.15	**-3.68**
family	Deferribacteraceae	1.73%	0.40%	0.23%	-2.11	-2.91
genus	Mucispirillum	1.73%	0.40%	0.23%	-2.11	-2.91
family	Streptococcaceae	0.24%	0.10%	0.05%	-1.31	**-2.39**
family	Incertae Sedis XIV	0.11%	9.00%	0.02%	6.37	-2.25
genus	Blautia	0.11%	9.00%	0.02%	6.37	-2.25
genus	Streptococcus	0.13%	0.05%	0.04%	-1.43	**-1.84**
genus	Lawsonia	0.39%	0.57%	0.12%	0.55	-1.69
genus	Oscillibacter	1.39%	0.77%	0.44%	-0.86	**-1.67**
family	Ruminococcaceae	3.18%	2.00%	1.06%	-0.67	**-1.59**
family	Coriobacteriaceae	0.45%	0.21%	0.17%	-1.12	**-1.44**
genus	Odoribacter	0.25%	0.37%	0.12%	0.56	-1.12
family	Desulfovibrionaceae	0.71%	0.95%	0.33%	0.41	-1.09
genus	Desulfovibrio	0.31%	0.35%	0.21%	0.17	-0.59
family	Helicobacteraceae	3.13%	1.13%	2.08%	-1.47	-0.59
family	Rikenellaceae	3.62%	7.30%	2.43%	1.01	**-0.57**
genus	Helicobacter	3.05%	1.12%	2.07%	-1.44	-0.56
genus	Rikenella	0.21%	0.38%	0.14%	0.88	-0.51
family	TM7 sub sub sub	0.02%	0.15%	0.02%	2.65	-0.45
genus	TM7 genera incertae sedis	0.02%	0.15%	0.02%	2.65	-0.45
genus	Escherichia/Shigella	0.03%	0.10%	0.02%	1.64	-0.39
genus	Alistipes	1.62%	4.64%	1.37%	1.52	-0.25
family	Enterococcaceae	9.05%	4.34%	7.63%	-1.06	**-0.25**
genus	Enterococcus	9.01%	4.33%	7.61%	-1.06	**-0.24**
family	Enterobacteriaceae	0.03%	0.11%	0.03%	1.77	-0.06
family	Porphyromonadaceae	3.99%	8.77%	5.34%	1.14	0.42
family	Lactobacillaceae	5.32%	7.14%	8.15%	0.42	**0.62**
genus	Lactobacillus	5.32%	7.14%	8.15%	0.42	**0.62**
family	Bacteroidaceae	12.71%	13.78%	19.88%	0.12	0.65
genus	Bacteroides	12.71%	13.78%	19.88%	0.12	0.65
genus	Parabacteroides	0.59%	0.97%	1.01%	0.71	0.77
genus	Barnesiella	0.24%	0.29%	0.65%	0.31	**1.46**
family	Lachnospiraceae	6.50%	14.57%	27.97%	1.17	**2.11**
family	Alcaligenaceae	0.22%	0.04%	0.00%	-2.41	na
genus	Parasutterella	0.21%	0.04%	0.00%	-2.54	na

Chapter 7

Summary and final considerations



Chapter 7

Summary and final considerations

Summary

A vast diversity of microbes, collectively called the intestinal microbiota, colonizes the gastrointestinal tract. Microbiota are vital for shaping the immune system and profoundly affect the balance between pro-inflammatory T helper (Th)1 and Th17 cells and anti-inflammatory regulatory T (Treg) cells, both at mucosal surfaces and systemically [1, 2]. A disturbance of the Th17/Treg balance is believed to promote autoimmunity [1]. Since microbiota greatly affect this balance, exposure to deranged intestinal microbiota may be a critical factor in the development of autoimmune arthritis. Several studies have shown that the intestinal microbiota of rheumatoid arthritis (RA) patients is disturbed [3-6]. In addition, several studies in mouse models suggest that arthritis is strongly affected by the intestinal microbiome [4, 5, 7-9]. In this thesis, we aimed to dissect the relevance of intestinal microbiota and intestinal mucosal immunity in the development of experimental arthritis.

In **Chapter 2**, evidence supporting the involvement of commensal microbiota in RA is reviewed. In addition, we discuss the potential role of Toll-like receptors (TLRs) in modulating intestinal T cell responses as a trigger for autoimmune arthritis. TLRs recognize microbe-associated molecular patterns (MAMPs) which are shared by many organisms. In addition, TLR activation is known to promote the interaction between antigen-presenting cells (APCs) and T cells, through upregulation of MHC class II molecules and costimulatory molecules. Furthermore, TLR activation profoundly affects the cytokine environment, thereby determining the direction of T cell differentiation. Multiple studies showed that TLR activation has a strong effect on intestinal T cell subsets. This makes TLRs interesting molecules to study in the context of intestinal T cell immunity and may offer new perspectives for modulation of Th cell response in RA patients.

Interleukin-1 receptor antagonist (IL-1Ra) is a natural inhibitor of the IL-1 receptor. IL-1Ra deficient mice are susceptible to the development of autoimmune diseases such as arthritis, diabetes and encephalomyelitis [10-14]. This indicates that IL-1Ra plays an important role in protection against autoimmunity. Previous studies have shown that arthritis in IL-1Ra$^{-/-}$ mice depends on T cells and IL-17 [11, 15]. In addition, an earlier study from our department showed that arthritis in IL-1Ra$^{-/-}$ mice is diminished under germ-free (GF) conditions [8], which suggests a role for microbiota in the induction of autoimmunity in these mice. We wanted to investigate the role of IL-1Ra in regulation of the intestinal microbiota and the involvement of mucosal immune responses in arthritis development in IL-1Ra$^{-/-}$ mice. Therefore, we characterized the intestinal microbiota and the underlying intestinal immune responses in IL-1Ra$^{-/-}$ mice (**Chapter 3**). We demonstrated

that the intestinal microbial diversity and richness is reduced in IL-1Ra$^{-/-}$ mice compared to wild-type mice. In addition, specific taxonomic alterations characterized by overrepresented *Helicobacter* and underrepresented *Ruminococcus* and *Prevotella* could be observed. Our data suggest that IL-1Ra plays an important role in regulating the intestinal microbiota diversity and composition.

The aberrant microbiota in IL-1Ra$^{-/-}$ mice specifically induced IL-17 production by intestinal lamina propria (LP) lymphocytes. Interestingly, transfer of IL-1Ra$^{-/-}$ microbiota to WT mice clearly potentiated IL-17 production by LP cells. In addition, intestinal as well as systemic Th17 levels were strongly reduced in GF IL-1Ra$^{-/-}$ mice. These findings indicate that IL-1Ra$^{-/-}$ microbiota promotes the development of intestinal and extra-intestinal Th17 cells during arthritis (**Figure 1**). To further determine the role of intestinal microbiota as a trigger for arthritis in IL-1Ra$^{-/-}$ mice, we partially depleted the intestinal microbiota by treating mice with a broad-spectrum cocktail of antibiotics. Treatment with antibiotics for 1 week strongly suppressed arthritis for up to 6 weeks after ceasing antibiotics. Interestingly, colonization of the antibiotic-treated mice with segmented filamentous bacteria (SFB), a potent Th17 inducing gut bacteria, resulted in exacerbation of arthritis. To determine which subset of the IL-1Ra$^{-/-}$ microbiota triggers arthritis we compared the effects of several relatively selective antibiotics on arthritis. Among the tested antibiotics, tobramycin treatment resulted in a strong reduction of the genera *Helicobacter*, *Flexispira*, *Clostridium* and *Dehalbacterium* and significantly suppressed arthritis. Previous studies form our department showed that TLR4 deficiency protected IL-1Ra$^{-/-}$ mice from severe arthritis [8]. Our analysis of the microbiota in this thesis (**Chapter 3**) showed that the aberrant microbiota observed in IL-1Ra$^{-/-}$ mice was partially restored in IL-1Ra$^{-/-}$TLR4$^{-/-}$ mice. Furthermore, LP cells from IL-1Ra$^{-/-}$TLR4$^{-/-}$ mice produced significantly less IL-1β, IL-23 and IL-6. This implies that TLR4 plays a significant role in the intestinal production of cytokines involved in Th17 differentiation. In **Chapter 3**, we identified an interplay between IL-1Ra, intestinal microbiota, TLR4 and intestinal T cells that may contribute to the development of autoimmune arthritis in mice (**Figure 1**). Intervening in this interplay might provide new opportunities to control the development or the progression of arthritis.

While multiple studies suggest a role of intestinal microbiota in promoting the development of autoimmune arthritis, it is not clear if perturbations in the intestinal microbiota precede the development of clinical arthritis or are merely a consequence of disease. Therefore we analyzed the intestinal microbiota of DBA1/J mice before and after immunization with collagen type II (CII, **Chapter 4**). We show that immunization with CII induces marked changes in the intestinal microbiota. While induction of collagen induced arthritis (CIA) did not affect bacterial richness and diversity, it did result in profound compositional changes in the microbiota. For instance, the abundance of the phylum Bacteroidetes,

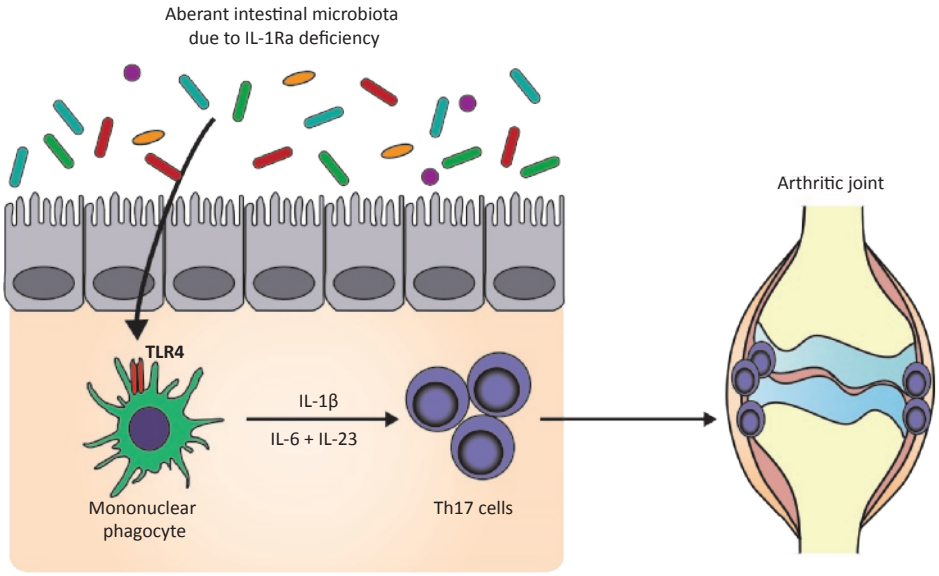


Figure 1. Aberrant intestinal microbiota in IL-1Ra deficient mice promotes the production of IL-1β, IL-6 and IL-23 by mononuclear phagocytes in the lamina propria via activation of Toll-like receptor TLR4. This results in increased differentiation of intestinal Th17 cells which contribute to development and progression of arthritis.

specifically families S24-7 and Bacteroidaceae was reduced, whereas families belonging to phylum Firmicutes and Proteobacteria, such as Ruminococcaceae, Lachnospiraceae and Desulfovibrinocaceae, expanded following a single injection with CII. Our data strongly suggests that alterations of the intestinal microbiota occur in the induction phase of inflammatory arthritis and precede the clinical manifestation of CIA.

We further examined whether the preclinical phase of experimental arthritis is accompanied with early immune activation. A significant increase in serum amyloid A (SAA) serum levels could be observed post-immunization compared to naive mice. In addition, the percentage of Th17 cells was significantly higher in joint-draining lymph nodes of immunized mice compared to naive mice. These data suggest that the preclinical phase of CIA is characterized by increased SAA serum levels and local Th17 cells.

Furthermore, we show that partial depletion of the intestinal microbiota with broad-spectrum antibiotics during ongoing CIA significantly reduced intestinal Th17 cells and attenuated arthritis. Interestingly, the abundance of Th17 cells in the intestinal lamina propria of CIA mice showed a striking correlation with arthritis severity, supporting the link between the microbiota-induced intestinal Th17 cells and autoimmune arthritis. While antibiotic treatment affected intestinal and local Th17 cell levels, antibiotic treatment did not affect the CII-specific antibody response. In addition, treatment with broad-spectrum antibiotics did not affect severity of serum-transfer arthritis. In contrast to the CIA model, serum-transfer arthritis is a T cell independent model [16, 17].

These data suggest that elimination of intestinal microbiota during T cell-mediated experimental arthritis can attenuate Th17-driven disease processes, whereas the antibody-mediated effector phase of arthritis does not depend on the intestinal microbiota.

Because the preclinical phase of CIA is characterized by marked changes in the intestinal microbiota (**Chapter 4**), we investigated whether the inductive, preclinical phase of experimental arthritis is accompanied by mucosal immune activation (**Chapter 5**). Our studies in **Chapter 5** showed that mice 21 days post immunization with CII, before the onset of arthritis, had significantly increased levels of IFNγ, IL-17, GM-CSF, IL-22 and TNFα expressing $CD4^+$ cells in SI-LP. In addition, the mesenteric lymph nodes (mLNs) of immunized mice contained significantly more GM-CSF and IL-17 producing CD4+ T cells compared to naive mice. Furthermore, Th17 cells from the SI-LP and mLNs of immunized mice more often co-expressed IL-22 than the Th17 cells from naive mice. This study suggests that the preclinical phase of CIA is indeed characterized by the activation of intestinal immunity.
Inhibition of IL-17 has been shown to suppress arthritis in mice, which suggests that IL-17 is a important cytokine in arthritis development [18, 19]. However, Th17 cells produce several other proinflammatory cytokines such as IL-22, TNFα and GM-CSF. To study the role of Th17 cells, beyond the role of IL-17, in arthritis development conditional Th17-deficient mice were utilized. These Th17-deficient mice have a specific reduction of Th17 cells as demonstrated in SI-LP and Peyer's patches. However, total levels of IL17A, produced by non-Th17 cells, were unaffected. Importantly, arthritis severity was markedly reduced in Th17-deficient mice. In addition, antigen-specific IL-17A, IL-17F and GM-CSF production was significantly reduced in joint draining lymph nodes of Th17-deficient mice.
Because the induction of Th17 cells in the body is highly affected by the intestinal microbiota, we hypothesized that the involvement of Th17 cells in arthritis depends on the microbial status of the host. To test this hypothesis, we replaced the intestinal microbiota of SFB-positive mice with SFB-free microbiota. SFB are known to be potent inducers of intestinal Th17 differentiation [20]. Interestingly, no significant effect of the Th17 deficiency on arthritis severity could be observed in the SFB free mice. This suggests that Th17 cells are only able to contribute to the pathogenesis of arthritis when specific Th17-inducing bacteria are present.

Our studies add to the previous evidence that the development and progression of arthritis is strongly affected by intestinal microbiota. Therefore, modulation of the intestinal microbiota may represent an interesting approach to suppress autoimmunity. Prebiotics, defined as non-digestible carbohydrates that selectively stimulate the growth and activity of beneficial microorganisms,

provide a relatively non-invasive approach to modulate the intestinal microbiota. In **Chapter 6,** we assessed the efficacy of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) as a therapeutic approach for T cell-dependent experimental arthritis in IL-1Ra$^{-/-}$ mice. We show that a prebiotic diet containing 2.5% scGOS/lcFOS significantly altered the composition of the intestinal microbiota. A prominent effect of the scGOS/lcFOS diet was a highly significant increase in the family Lachnospiraceae. In line with previous studies, scGOS/lcFOS diet resulted in increased abundance of the genera *Lactobacillus.* The genera *Enterococcus* and *Clostridium* were markedly decreased by scGOS/lcFOS dietary supplementation. Furthermore, we show that prebiotic diet containing 5% scGOS/lcFOS significantly increases the bone mineral density. To determine the therapeutic efficacy of scGOS/lcFOS in treatment of arthritis, we fed IL-1Ra$^{-/-}$ mice with ongoing arthritis with a 1% or 2.5% scGOS/lcFOS containing diet or a control diet. The mice receiving the 2.5% scGOS/lcFOS showed a trend towards reduced arthritis severity. Aiming to maximize the observed effects, the experiment was replicated with IL-1Ra$^{-/-}$ mice receiving 2.5% or 5% scGOS/lcFOS. However, this study showed no effect on arthritis scores at all. In addition, analysis of Th cell subsets showed no effect of the prebiotic diet on intestinal and joint-associated Th cell subsets. Altogether, this study suggests that dietary scGOS/lcFOS supplementation is able to promote presumably healthy gut microbiota and improve bone mineral density, but not inflammation, in arthritis-prone mice

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Conclusion and final considerations

In this thesis, we aimed to dissect the relevance of intestinal microbiota and intestinal mucosal immunity in the development of experimental arthritis. We have demonstrated that aberrant intestinal microbiota potentiate intestinal Th17 differentiation and thereby contribute to the development of autoimmune arthritis in mice. Moreover, our data suggests that TLR4 is involved in mucosal Th17 induction. Interventions aimed at inhibiting the TLR4-mediated mucosal T cell response may represent a novel approach to control RA. In addition, we provided evidence that the preclinical phase of CIA is characterized by marked changes in the intestinal microbiota and activation of mucosal immunity before any clinical symptoms. While multiple studies have shown that the intestinal microbiota is perturbed in early RA patients, longitudinal studies are needed to investigate if the preclinical phase of RA is accompanied with shifts in intestinal microbiota and mucosal immune activation. A better understanding of the preclinical phase of RA and the involvement of mucosal immunity in this phase will help identify new therapeutic or even preventive strategies. Moreover, our data suggests that intestinal microbiota can influence inflammation and promote arthritis even after its onset. This makes modulation of the intestinal microbiota

an interesting approach to suppress autoimmunity. Even though antibiotic treatment is effective in dampening inflammatory arthritis in mice, the long-term use should be avoided in patients, because antibiotic treatment will result in the depletion of beneficial bacteria and will increase the occurrence of antibiotic-resistant bacteria. Therefore, alternative, more specific ways to modulate the intestinal microbiota of patients should be considered. While in our study the prebiotic treatment failed to significantly reduce inflammation in mice, the use of prebiotics in modulating the intestinal microbiota remains an interesting field of investigation. In addition, using probiotics to modulate the intestinal microbiota of RA patients is a promising way to influence disease activity. Two clinical studies with *Lactobacillus casei* showed improved disease activity scores and decreased pro-inflammatory cytokine and C-reactive protein serum levels after prebiotic treatment [21, 22]. Another study, using a capsule containing *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium bifidum,* showed that this mixture improved disease activity scores and C-reactive protein serum levels in RA patients[23]. However, another study using a combination of *Lactobacillus rhamnosus* and *Lactobacillus reuteri* showed no clinical effect on RA [24]. Further studies with higher numbers of patients and longer evaluation times are needed to confirm a positive effect of probiotics on disease outcome.

Another attractive therapeutic strategy to modulate the intestinal immune response, and potentially treat autoimmune diseases such as RA, is with bacterial-derived immune modulating products. One well studied bacterial-derived molecule is Polysaccharide A (PSA) from *Bacteroides fragilis. B.fragilis*–derived PSA induces a strong Treg cell response and protects mice from developing colitis and experimental autoimmune encephalomyelitis (EAE) [25, 26]. *In vitro* studies have shown that PSA is also capable to induce Treg cell differentiation in humans, which makes the use of PSA in treating autoimmune diseases promising [27].

Gut-microbiota secrete large amounts of metabolites which affect host immunity as well. For instance, the short-chain fatty acids (SCFAs) butyrate and propionate which are products of bacterial fermentation of dietary fiber, are known to induce differentiation of Tregs [28-30]. Mice fed specific diets designed to release high levels of acetate and butyrate following bacterial fermentation had markedly reduced frequencies of autoreactive T cells, increased numbers of Treg cells and diabetes was ameliorated [31]. Studying the immunomodulatory properties of microbial metabolites could provide novel therapeutic strategies for treating autoimmune arthritis.

Furthermore, we showed that in the absence of SFB, Th17 involvement is not evident anymore. This suggests that Th17 cells are only able to mediate arthritis pathogenesis in the context of specific microbiota. A recent study showed that colonization with SFB triggers systemic autoimmunity in mice by selectively expanding Th17 cells that co-express SFB and auto-antigen specific T cell receptors (TCRs) [32]. Favoring the expansion of dual TCR expressing T cells

could be a mechanism by which SFB contributes to the development of autoimmunity, as it has been suggested that self-reactive T cells expressing an additional non-self TCR can escape thymic deletion [32, 33]. T cell epitope mimicry between microbial- and self-peptides is another suggested mechanism implicated in the development of autoimmunity. T cell epitopes of two auto-antigens, N-acetylglucosamine-6-sulfatase (GNS) and filamin A (FLNA), were recently shown to share homology with epitopes from proteins of the gut bacteria *Prevotella* spp. and *Parabacteroides* spp [34]. Both GNS and FLNA were highly expressed in inflamed RA synovium [34]. In addition, T cells from patients who showed reactivity to these self-antigens often also responded to the corresponding bacterial peptides [34]. Furthermore, citrullinated vinculin, an auto-antigen present in inflamed RA joints is targeted by ACPA and $CD4^+$ T cells. These T cells recognize an epitope which contains the sequence DERAA, a sequence which is also found in many microbes [35]. These studies provide strong evidence in favor of the hypothesis that T cells activated by microbial peptides in the intestine may home to inflamed synovium and cross-react with homologous self-antigens. Another possibility is that gut-microbiota-activated Th17 cells migrate to the joints where they promote the differentiation and activation of self-reactive Th17 cells by changing the local cytokine environment. A better understanding of how microbiota-induced Th17 cells promote the development of autoimmunity might offer new perspectives for manipulating the Th17 response in RA patients.

Another challenge will be to identify and characterize specific Th17-inducing bacteria in RA patients. This would make the microbiome profile of a patient a valuable predictive biomarker for the efficacy of Th17-targeted therapies. In addition, targeting these specific Th17-inducing bacteria could be an interesting way to block the intestinal induction of disease promoting Th17 cells in RA. Another approach would be to target host immune mechanisms triggered by these bacteria. Moreover, it would be interesting to identify Treg cell-inducing commensal bacteria, as these cells might be able to dampen the excessive Th17 responses in RA patients. Understanding the exact mechanisms involved in the microbiome-driven Th17/Treg balance in RA patients may help identify novel therapeutic strategies for inflammatory arthritis.

References

1. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75-84.
2. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science (New York, NY). 2012;336(6086):1268-73.
3. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife. 2013;2:e01202.
4. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. Genome medicine. 2016;8(1):43.
5. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine. Arthritis & rheumatology (Hoboken, NJ). 2016;68(11):2646-61.
6. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med. 2015;21(8):895-905.
7. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity. 2010;32(6):815-27.
8. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. The Journal of clinical investigation. 2008;118(1):205-16.
9. Marietta EV, Murray JA, Luckey DH, Jeraldo PR, Lamba A, Patel R, et al. Suppression of Inflammatory Arthritis by Human Gut-Derived Prevotella histicola in Humanized Mice. Arthritis & rheumatology (Hoboken, NJ). 2016;68(12):2878-88.
10. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. The Journal of experimental medicine. 2000;191(2):313-20.
11. Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(10):5986-90.
12. Shepherd J, Little MC, Nicklin MJ. Psoriasis-like cutaneous inflammation in mice lacking interleukin-1 receptor antagonist. The Journal of investigative dermatology. 2004;122(3):665-9.
13. Matsuki T, Horai R, Sudo K, Iwakura Y. IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions. The Journal of experimental medicine. 2003;198(6):877-88.
14. Matsuki T, Nakae S, Sudo K, Horai R, Iwakura Y. Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis. International immunology. 2006;18(2):399-407.
15. Koenders MI, Devesa I, Marijnissen RJ, Abdollahi-Roodsaz S, Boots AM, Walgreen B, et al. Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice. Arthritis and rheumatism. 2008;58(11):3461-70.
16. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity. 1999;10(4):451-61.
17. Kyburz D, Corr M. The KRN mouse model of inflammatory arthritis. Springer Semin Immunopathol. 2003;25(1):79-90.
18. Koenders MI, Lubberts E, Oppers-Walgreen B, van den Bersselaar L, Helsen MM, Di Padova FE, et al. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. Am J Pathol. 2005;167(1):141-9.

19. Lubberts E, Koenders MI, Oppers-Walgreen B, van den Bersselaar L, Coenen-de Roo CJ, Joosten LA, et al. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis and rheumatism. 2004;50(2):650-9.
20. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139(3):485-98.
21. Alipour B, Homayouni-Rad A, Vaghef-Mehrabany E, Sharif SK, Vaghef-Mehrabany L, Asghari-Jafarabadi M, et al. Effects of Lactobacillus casei supplementation on disease activity and inflammatory cytokines in rheumatoid arthritis patients: a randomized double-blind clinical trial. Int J Rheum Dis. 2014;17(5):519-27.
22. Vaghef-Mehrabany E, Alipour B, Homayouni-Rad A, Sharif SK, Asghari-Jafarabadi M, Zavvari S. Probiotic supplementation improves inflammatory status in patients with rheumatoid arthritis. Nutrition. 2014;30(4):430-5.
23. Zamani B, Golkar HR, Farshbaf S, Emadi-Baygi M, Tajabadi-Ebrahimi M, Jafari P, et al. Clinical and metabolic response to probiotic supplementation in patients with rheumatoid arthritis: a randomized, double-blind, placebo-controlled trial. Int J Rheum Dis. 2016;19(9):869-79.
24. Pineda Mde L, Thompson SF, Summers K, de Leon F, Pope J, Reid G. A randomized, double-blinded, placebo-controlled pilot study of probiotics in active rheumatoid arthritis. Med Sci Monit. 2011;17(6):Cr347-54.
25. Ochoa-Reparaz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal Bacteroides fragilis protects against CNS demyelinating disease. Mucosal immunology. 2010;3(5):487-95.
26. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell host & microbe. 2012;12(4):509-20.
27. Telesford KM, Yan W, Ochoa-Reparaz J, Pant A, Kircher C, Christy MA, et al. A commensal symbiotic factor derived from Bacteroides fragilis promotes human CD39(+)Foxp3(+) T cells and Treg function. Gut microbes. 2015;6(4):234-42.
28. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science (New York, NY). 2013;341(6145):569-73.
29. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50.
30. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5.
31. Marino E, Richards JL, McLeod KH, Stanley D, Yap YA, Knight J, et al. Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. Nature immunology. 2017;18(5):552-62.
32. Bradley CP, Teng F, Felix KM, Sano T, Naskar D, Block KE, et al. Segmented Filamentous Bacteria Provoke Lung Autoimmunity by Inducing Gut-Lung Axis Th17 Cells Expressing Dual TCRs. Cell host & microbe. 2017;22(5):697-704.e4.
33. Auger JL, Haasken S, Steinert EM, Binstadt BA. Incomplete TCR-beta allelic exclusion accelerates spontaneous autoimmune arthritis in K/BxN TCR transgenic mice. European journal of immunology. 2012;42(9):2354-62.
34. Pianta A, Arvikar SL, Strle K, Drouin EE, Wang Q, Costello CE, et al. Two rheumatoid arthritis-specific autoantigens correlate microbial immunity with autoimmune responses in joints. The Journal of clinical investigation. 2017;127(8):2946-56.

35. van Heemst J, Jansen DT, Polydorides S, Moustakas AK, Bax M, Feitsma AL, et al. Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis. Nature communications. 2015;6:6681.

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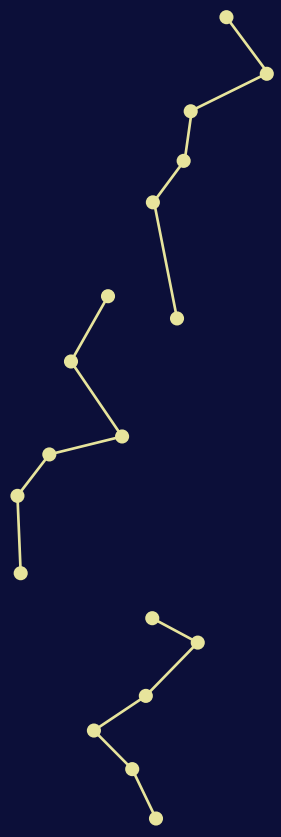

Chapter 8

Nederlandse samenvatting

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Nederlandse samenvatting

Reumatoïde artritis

Reumatoïde artritis (RA) is een van de meest voorkomende auto-immuunziektes en treft ongeveer 0.5 – 1% van de wereldbevolking. RA wordt gekenmerkt door chronische ontsteking van het synovium, ook wel het gewrichtskapsel genoemd. Deze chronische ontsteking kan uiteindelijk leiden tot afbraak van kraakbeen en bot. Dit belemmert de bewegingsvrijheid van het gewricht en veroorzaakt pijn. In het ontstoken weefsel bevinden zich grote hoeveelheden ontstekingscellen, o.a. monocyten, macrofagen, en B en T cellen. Deze ontstekingscellen produceren grote hoeveelheden cytokines en chemokines (signaaleiwitten) die er voor zorgen dat er nog meer ontstekingscellen geactiveerd en aangetrokken worden. De geactiveerde ontstekingscellen kunnen uiteindelijk ook het kraakbeen en bot aantasten. Alhoewel het onduidelijk is wat de precieze oorzaak van RA is, gaat men er van uit dat zowel genetische als omgevingsfactoren bijdragen aan het ontstaan van de ziekte. Roken is een van de omgevingsfactoren die een rol lijkt de spelen in het ontstaan van RA. Daarnaast worden micro-organismen in onder andere de longen, mond en darmen in verband gebracht met de ontwikkeling van RA.

Immuunsysteem en darmbacteriën

In en op ons lichaam bevindt zich een grote diversiteit aan micro-organismen (o.a. bacteriën en schimmels). Al deze micro-organismen (en hun genen) worden samen het microbioom genoemd. Het darmstelsel is veruit het dichtst bevolkt en bevat voornamelijk grote hoeveelheden bacteriën. Darmbacteriën zijn van groot belang voor de vertering van bepaalde voedingsstoffen en ze produceren belangrijke vitamines en nutriënten. Daarnaast spelen ze een belangrijke rol in de ontwikkeling van het immuunsysteem. Er zijn echter ook slechte (pathogene) bacteriën die infecties veroorzaken. Normaliter beschermt het immuunsysteem ons lichaam tegen pathogene bacteriën en zorgt voor een gezonde balans tussen tolerantie van de goede bacteriën en afweer tegen de slechte bacteriën. T helper (Th) cellen vormen een belangrijk onderdeel van dit beschermingsmechanisme en kunnen worden onderverdeeld in Th1, Th2 en Th17 cellen. Daarnaast zijn er de regulatoire T cellen (Treg) die de immuunreactie kunnen onderdrukken. Th cellen zijn niet alleen betrokken bij bescherming tegen infecties maar ook bij de ontwikkeling van auto-immuunziektes zoals RA. Th cellen zijn in grote aantallen aanwezig in het ontstekingsweefsel van RA patiënten. Het belang van T cellen in RA wordt ondersteund door verschillende dierstudies die onder andere laten zien dat je artritis kan overdragen door enkel Th cellen van een zieke muis te injecteren in een gezonde muis.

Darmbacteriën en RA
De ontwikkeling van auto-immuunreacties beginnen jaren voordat een patiënt klachten krijgt. Het ontbreken van de gewrichtsontsteking in deze beginfase van RA, suggereert dat de ziekte ergens anders begint. Steeds meer studies laten zien dat de darmen en de bacteriën die zich daar bevinden mogelijk een rol spelen in het ontstaan van RA. Zo hebben studies in muizen laten zien dat de ontwikkeling van auto-immuunziektes afhankelijk is van de aanwezigheid van microbiota. Kiemvrije muizen, muizen die helemaal geen micro-organismen bij zich dragen en in een steriele omgeving leven, zijn namelijk deels beschermd tegen auto-immuunziektes zoals experimentele artritis (een diermodel voor RA). Kiemvrije muizen ontwikkelen een duidelijk minder ernstige vorm van de ziekte, met verminderde ontsteking en schade in het gewricht. Daarnaast hebben verschillende studies aangetoond dat de diversiteit en samenstelling van het darmmicrobioom verstoord is in RA patiënten. Bepaalde bacteriesoorten (o.a. *Prevotella copri*) lijken meer voor te komen in RA patiënten, terwijl andere bacteriën juist minder aanwezig zijn. Deze studies suggereren een link tussen darmbacteriën en de ziekte RA, maar het mechanisme hierachter wordt nog niet goed begrepen.

Doel van dit proefschrift

In dit proefschrift hebben we onderzoek gedaan naar de rol van darmbacteriën in de ontwikkeling van auto-immuun artritis in muizen. Het doel hierbij was om mechanismen te identificeren waarmee bacteriën het immuunsysteem ontregelen en het ontstaan van auto-immuun artritis bevorderen. In **hoofdstuk 2** worden studies samengevat die er op wijzen dat T cellen in de darmen (met name Th17 cellen) door darmbacteriën worden geactiveerd en op die manier een rol spelen in de ontwikkeling van RA. Daarnaast wordt de mogelijke rol van Toll-like receptoren (TLRs) in dit proces besproken. TLRs zijn eiwitten die bacteriën herkennen en als reactie daarop het immuunsysteem activeren. Dit doen ze onder andere door de interactie tussen verschillende ontstekingscellen te bevorderen en door de productie van ontstekingseiwitten (cytokines). Verschillende studies hebben laten zien dat TLR activatie een sterk effect heeft op verschillende typen T cellen in de darmen. Dit maakt TLRs interessante moleculen om te onderzoeken in de context van T cel activatie in RA patiënten.

Interleukin-1 receptor antagonist (IL-1Ra) is een natuurlijke remmer van de cytokine IL-1. Muizen die het IL-1Ra eiwit missen zijn vatbaar voor de ontwikkeling van auto-immuunziektes zoals auto-immuun artritis. Dit suggereert dat IL-1Ra een belangrijke rol speelt in de bescherming tegen auto-immuniteit. Voorgaande studies hebben laten zien dat artritis in IL-1Ra deficiënte (IL-1Ra$^{-/-}$) muizen afhankelijk is van T cellen en IL-17. Daarnaast is aangetoond dat kiemvrije IL-1Ra$^{-/-}$ muizen

een mindere ernstig ziektebeeld vertonen. Dit laatste suggereert dat bacteriën een belangrijke rol spelen in de inductie van artritis in deze muizen. Om te onderzoeken wat het ontbreken van het IL-1Ra eiwit betekent voor het darmmicrobioom en het immuunsysteem, hebben we de samenstelling van de darmmicrobiota van IL-1Ra$^{-/-}$ muizen onderzocht (Hoofdstuk 3). We laten zien dat IL-1Ra een belangrijke rol speelt in het onderhoud van de natuurlijke diversiteit en samenstelling van het darmmicrobioom. De verstoring van de darmbacteriën lijkt met name de productie van IL-17 in de darmen te bevorderen. Daarnaast laten we zien dat kiemvrije IL-1Ra$^{-/-}$ muizen veel minder IL-17 producerende Th17 cellen in zowel de darmen als in de rest van het lichaam hebben. Dit suggereert dat de darmbacteriën van IL-1Ra$^{-/-}$ muizen een belangrijke rol spelen in het induceren van Th17 cellen. Toedienen van een antibiotica cocktail, die een groot deel van de darmbacteriën doodt, zorgde voor een sterke onderdrukking van de artritis. Het toedienen van Th17 inducerende bacteriën na antibiotica behandeling deed dit effect teniet. Behandeling met tobramycine, een antibiotica dat specifiek gramnegatieve bacteriën bestrijdt, resulteerde in een vermindering van de artritis verschijnselen. Dit wijst er op dat gramnegatieve bacteriën mogelijk een rol spelen in de ontwikkeling van artritis. Vervolgens hebben we de rol van TLR4 bestudeerd. In eerdere studies is aangetoond dat IL-1Ra$^{-/-}$ muizen die ook het TLR4 eiwit missen een minder ernstige vorm van artritis ontwikkelden. Wij laten zien dat in deze TLR4 deficiënte IL-1Ra muizen het darmmicrobioom deels hersteld is. Daarnaast laten we zien dat TLR4 een belangrijke rol speelt in de productie van de pro-inflammatoire cytokines (IL-1, IL-23 en IL-6) in de darmen. Dit impliceert dat TLR4 een belangrijke rol speelt in de inductie van Th17 cellen in de darmen. Samengevat hebben we in **hoofdstuk 3** een interactie geïdentificeerd tussen IL-1Ra, darmbacteriën, TLR4 en T cellen die mogelijk bijdraagt aan de ontwikkeling van artritis in muizen. Ingrijpen in deze interactie biedt nieuwe mogelijkheden om de ontwikkeling en progressie van artritis te remmen.

Meerdere studies suggereren dat darmbacteriën een rol spelen in de ontwikkeling van auto-immuun artritis in muizen. Het is echter niet duidelijk of verstoring van het darmmicrobioom vooraf gaat aan het ontstaan van de gewrichtsontsteking. Daarom hebben we de darmbacteriën van muizen geanalyseerd voor en na het induceren van collageen geïnduceerde artritis (CIA) in **hoofdstuk 4**. We tonen aan dat één enkele injectie met collageen type II (CII) een verschuiving in de samenstelling van het darmmicrobioom veroorzaakt. Deze verandering was al aanwezig voordat er tekenen van gewrichtsontsteking zichtbaar waren. Deze preklinische fase ging gepaard met een verhoogde expressie van de SAA eiwitten (ontstekingseiwitten). Daarnaast was het percentage Th17 cellen aanzienlijk hoger in de lymfeknopen van muizen geïmmuniseerd met CII.

Daarnaast laten we zien dat behandeling met breedspectrum antibiotica tijdens CIA leidt tot een sterke vermindering van het aantal Th17 cellen in de darmen. Ook wordt de artritis sterk onderdrukt door deze behandeling. Interessant genoeg zagen we een sterke correlatie tussen de hoeveelheid Th17 cellen in de darmen en de ernst van de gewrichtsontsteking. Dit ondersteunt het idee dat er een link is tussen microbioom-geïnduceerde Th17 cellen en auto-immuun artritis. Daarentegen had de antibioticabehandeling geen effect op de productie van CII specifieke antilichamen. Daarnaast had behandeling met een breedspectrum antibiotica ook geen effect op de ernst van serum-transfer artritis. In tegenstelling tot CIA, is serum-transfer artritis een artritismodel dat onafhankelijk is van T cellen. Deze resultaten suggereren dat eliminatie van de darmbacteriën tijdens (T cel afhankelijke) artritis Th17-gedreven ziekteprocessen kan onderdrukken. Ziekteprocessen gedreven door antilichamen niet beïnvloed lijken te worden door de darmbacteriën.

Omdat de preklinische fase van CIA gekarakteriseerd wordt door veranderingen in het darmmicrobioom hebben we onderzocht wat er met het immuunsysteem gebeurd in deze fase van artritis. In **hoofdstuk 5** laten we zien dat muizen 21 dagen na injectie met CII, maar voor de ontwikkeling van gewrichtsontsteking, aanzienlijk meer IFNγ, IL-17, GM-CSF, IL-22 en TNFα producerende Th cellen in de darmen en lymfeknopen hebben. Dit suggereert dat de preklinische fase van CIA gepaard gaat met activatie van het immuunsysteem in de darmen.
Meerdere studies hebben laten zien dat het remmen van IL-17 erg effectief is in het onderdrukken van artritis in muizen. Dit suggereert dat IL-17 een belangrijke rol speelt in de ontwikkeling van artritis. Th17 cellen produceren echter ook nog verschillende andere pro-inflammatoire cytokines zoals IL-22, TNFα en GM-CSF. Daarnaast wordt IL-17 ook door andere cellen (neutrofielen en mestcellen) geproduceerd. Om de rol van Th17 cellen tijdens de ontwikkeling van artritis te onderzoeken hebben we gebruik gemaakt van Th17 deficiënte muizen. Deze Th17 deficiënte muizen hadden een sterke vermindering van het aantal IL-17 producerend Th cellen in de darmen. IL-17 productie door andere cellen was onveranderd. Artritisverschijnselen waren sterk verminderd in de Th17 deficiënte muizen.
Aangezien de inductie van Th17 cellen in het lichaam sterk wordt beïnvloed door het darmmicrobioom, hebben we getest of de betrokkenheid van Th17 cellen in artritis afhangt van de microbiële status van de muis. Hiervoor hebben we het microbioom van SFB-positieve muizen vervangen met het microbioom van SFB-negatieve muizen. SFB (Segmented filamentous bacteria) zijn darmbacteriën waarvan bekend is dat ze Th17 differentiatie en proliferatie in de darm induceren. Interessant genoeg was er in de SFB-negatieve muizen geen effect merkbaar van de Th17 deficiëntie. Dit suggereert dat Th17 cellen alleen een bijdrage leveren aan het ziekteproces als specifieke Th17-activerende bacteriën aanwezig zijn.

Tenslotte hebben we onderzocht of modulatie van het darmmicrobioom een mogelijke aanpak zou kunnen zijn om auto immuunreacties te onderdrukken. Prebiotica zijn niet verteerbare koolhydraten die selectief de groei en activiteit van goede bacteriën in de darm stimuleren. Prebiotica zouden dus mogelijk gebruikt kunnen worden om het darmmicrobioom te moduleren. In **hoofdstuk 6** hebben we onderzocht of een mix van prebiotica, bestaande uit short-chain galacto-oligosaccharides en long-chain fructo-oligosaccharides (scGOS/lcFOS), een therapeutisch effect heeft op artritis in IL-1Ra$^{-/-}$ muizen. We laten zien dat een prebiotisch dieet dat 2.5% scGOS/lcFOS bevat een duidelijk effect heeft op het microbioom. We zagen onder andere een sterke toename in de familie Lachnospiraceae en het geslacht *Lactobacillus*. Daarentegen werden bacteriën behorende tot het Enterococcus en Clostridium geslacht duidelijk verminderd door het scGOS/lcFOS dieet. Daarnaast laten we zien dat een prebiotisch dieet met 5% scGOS/lcFOS zorgt voor een toename van de bot mineraaldichtheid. Om het therapeutisch effect op artritis te testen hebben we IL-1Ra$^{-/-}$ muizen met artritis voer gegeven waaraan 1% , 2.5% of geen scGOS/lcFOS was toegevoegd. De muizen die het voer met 2.5% scGOS/lcFOS kregen lieten een niet-significante trend zien van verminderde artritis. Analyse van de T cellen in deze muizen liet geen effect zien van het prebiotische dieet op de T cel populaties in zowel de darmen als in het gewricht. Deze studie laat zien dat scGOS/lcFOS de groei van specifieke darmbacteriën kan stimuleren en bot mineraaldichtheid kan verbeteren, maar dat het geen effect heeft op gewrichtsontsteking in muizen met artritis.

Conclusie

In dit proefschrift hebben we laten zien dat bacteriën en immuunreacties in de darmen een belangrijke rol spelen in de ontwikkeling van artritis in muizen. Bovendien hebben we aangetoond dat darmbacteriën Th17 cellen induceren en op die manier bijdragen aan de ontwikkeling van auto-immuun artritis. Modulatie van het darmmicrobioom en specifiek het bestrijden van Th17 inducerende bacteriën of het remmen van Th17 activatie in de darmen van patiënten, zou dan ook een interessante toekomstige therapie voor RA kunnen zijn.

List of publications

Rogier R, Wopereis H, Ederveen THA, Hartog A, Boekhorst J, van Hijum SAFT, Knol J, Garssen J, Walgreen B, Helsen MM, van der Kraan PM, van Lent PLEM., van de Loo FAJ, Koenders MI, Abdollahi-Roodsaz S. Supplementation of diet with non-digestible oligosaccharides alters the intestinal microbiota, but not arthritis development, in IL-1 receptor antagonist deficient mice. [Submitted for publication]

Evans-Marin H, **Rogier R**, Koralov SB, Julia Manasson, Roeleveld DM, van de Kraan PM, Scher JU, Koenders MI and Abdollahi-Roodsaz S. Involvement of T helper 17 cells in inflammatory arthritis depends on the host intestinal microbiota. Arthritis and Rheumatology (2018) *70 (12): 1971-1983.*

Rogier R, Evans-Marin H, Manasson J, van der Kraan PM, Walgreen B, Helsen MM, van den Bersselaar LA, van de Loo FAJ, van Lent PLEM, Abramson SB, van den Berg WB, Koenders MI, Scher JU and Abdollahi-Roodsaz S. Alteration of the intestinal microbiome characterizes preclinical inflammatory arthrtitis in mice and its modulatation attenuates established arthritis. Scientific reports (2017)7:15613.

Rogier R, Ederveen THA, Boekhorst J, Wopereis H, Scher JU, Manasson J, SJCM Frambach, Knol J, Garssen J, van der Kraan PM, Koender MI, van den Berg WB, van Hijum SAFT and Abdollahi-Roodsaz S. Aberrant intestinal microbiota due to IL-1 receptor antagonist deficiency promotes IL-17- and TLR4-dependent arthritis. Microbiome (2017) 5(1): 63.

Rogier R, Koenders MI and Abdollahi-Roodsaz S. Toll-Like Receptor Mediated Modulation of T Cell Response by Commensal Intestinal Microbiota as a Trigger for Autoimmune Arthritis. Journal of Immunology Research (2015) 2015:527696.

Curriculum Vitae

Rebecca Laurence Rogier werd op 15 november 1988 geboren te Oss. Na het behalen van haar VWO diploma aan het Mondriaan college in Oss in 2007, is zij Medische Biologie gaan studeren aan de Radboud Universiteit Nijmegen. Tijdens haar bachelor liep zij stage op de afdeling Cellulaire Dierfysiologie onder begeleiding van dr. Tamás Kozicz in de groep van Prof. dr. E.W. Roubos. In 2010 begon zij aan haar master Medical Biology, waarvoor zij stage liep of de afdeling Hematologie van het Raboud umc onder begeleiding van Jimmy Israël in de groep van dr. Bert van der Reijden. Daarnaast liep zij stage op de afdeling Experimentele Reumatologie van het Radboud umc onder begeleiding van dr. Shahla Abdollahi-Roodsaz in de groep van dr. Marije Koenders. In 2013 behaalde zij haar master diploma waarna zij begon als promovendus op de afdeling experimentele reumatologie opnieuw onder de begeleiding van dr. Shahla Abdollahi-Roodsaz en dr. Marije Koenders. Hier deed zij ruim vier jaar onderzoek naar de relevantie van darmbacteriën en immuunreacties in de darm tijdens de ontwikkeling van auto-immuun artritis. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Inmiddels is Rebecca sinds januari 2018 gaan werken als Quality Assucrance Officer bij Eurotrol in Ede.

Research data management

Findable
All (meta) data described in this thesis can be found at the department of Experimental Rheumatology of the Radboud university medical center.

Accesible
The (meta) data and protocols can be obtained on request from the department of Experimental Rheumatology of the Radboud university medical center. In addition, all 16S rRNA gene sequencing reads data are publicly available at the European Nucleotide Archive (ENA) database (http://www.ebi.ac.uk/ena).

Interoperable
The (meta) data are documented in English according to the FAIR priniciples.

Reusable:
The (meta) data shown in this thesis are sufficiently documented to be reusable.

Portfolio

Attended symposia

December 2013	New frontiers symposium RIMLS
April 2014	NVVI symposium Mucosal immunology: crossing borders
October 2014	New frontiers symposium RIMLS
March 2015	NVVI symposium Immunity and Science Fiction: the next 50 years in Immunology
March 2016	NVVI symposium The immune system strikes back

Attended (inter)national congresses

September 2013	Annual meeting BeTheCURE, Prague. *Poster presentation*.
February 2014	European workshop for rheumatology research (EWRR) 2014, Lisbon, *podium presentation*.
May 2014	RIMLS PhD retreat, Wageningen. *Poster presentation*.
September 2014	Annual congress Nederlandse vereniging voor reumatologie (NVR), Arnhem. *Podium presentation*.
December 2014	Annual congress of the Dutch society for immunology (Nederlandse vereniging voor immunologie, NNVI), Kaatsheuvel. *Podium presentation*.
September 2015	Annual congress Nederlandse vereniging voor reumatologie (NVR), Arnhem. *Podium presentation*.
December 2015	Annual congress of the Dutch society for immunology (Nederlandse vereniging voor immunologie, NNVI), Noordwijkerhout. *Podium presentation*.
February 2016	European workshop for rheumatology research (EWRR) 2016, York, *podium presentation*.
April 2016	RIMLS PhD retreat, Veldhoven. *Poster presentation*.

April 2016	International congress on autoimmunity 2016. Leipzig. *Podium presentation.*
May 2016	BeTheCure Tolerance workshop. Sigtuna.
December 2016	Annual congress Dutch and Britisch society for immunology (NVVI/BSI) 2016. Liverpool. *Poster presentation.*
February 2017	Annual meeting BeTheCURE, Prague. *Poster presentation*. *Podium presentation*.

Scholarship

- EULAR Travel bursary to attend EWRR (February 2014).
- Radboud Internationalization travel grant for outgoing PhD candidates to attend BSI/NVVI meeting (December 2016)

Courses

- Scientific Writing (3 ECTS)
- Opfriscursus Statistiek voor Promovendi (2 ECTS)
- Presenteren eigen onderzoek (1.5 ECTS)
- Loopbaanmanagement voor Promovendi (1 ECTS)
- Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK) (1.5 ECTS)

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Isabelle, mijn lieve zusje en beste vriendin. Jij bent de zorgzaamste persoon die ik ken, al sinds onze kindertijd maak jij je vooral druk om anderen. Ik kan me geen betere zus wensen. Hoewel het lastig was om te begrijpen waar mijn onderzoek over ging (iets met muizen en reuma toch?...) heb je toch ook zeker bijgedragen aan dit proefschrift, bedankt voor al je steun. Cees, heel erg bedankt voor je hulp met de layout van dit proefschrift, dankzij jou heeft dit boekje ook een omslag. David, mijn “kleine” broertje, fijn dat jij de boel in Oss een beetje in de gaten houdt nu je zussen het mooie Oss hebben verlaten. David en Amber, bedankt voor jullie gezelligheid en steun. Amber, ik beloof dat ik vanaf nu de muizen met rust laat.

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